

THE ROLE OF INFLAMMATION AND MATRIX PROTEINS IN AIRWAY REMODELLING

ANNA MEURONEN

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The role of inflammation and matrix proteins in airway remodelling

Anna Meuronen

Institute of Biomedicine, Anatomy
University of Helsinki
Helsinki, Finland

and

Department of Medicine, Division of Respiratory Diseases
Helsinki University Central Hospital
Helsinki, Finland

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Supervised by

Docent Annika Laitinen, MD, PhD
Institute of Biomedicine, Department of Anatomy
University of Helsinki
Helsinki, Finland

Professor Lauri A. Laitinen, MD, PhD, FRCP
Department of Medicine, Division of Respiratory Diseases
Helsinki University Central Hospital
Helsinki, Finland

Reviewed by

Professor Eeva Moilanen, MD, PhD
The Immunopharmacology Research Group
University of Tampere School of Medicine
Tampere, Finland

Professor Riitta Lahesmaa, MD, PhD
Turku Centre for Biotechnology
University of Turku
Turku, Finland

Opponent

Docent Hannu Kankaanranta, MD, PhD
Department of Respiratory Medicine
Seinäjäki Central Hospital, Seinäjoki, Finland
and
University of Tampere School of Medicine
Tampere, Finland

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*To mamma, mum
and auntie Eve*

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List of original publications

- I) **Meuronen A**, Majuri ML, Alenius H, Mäntylä T, Wolff H, Piirilä P, Laitinen A. Decreased cytokine and chemokine mRNA expression in BAL in asymptomatic smoking subjects. *Respiration* 2008;75:450-458
- II) Piirilä P, **Meuronen A**, Majuri ML, Luukkonen R, Mäntylä R, Wolff HJ, Nordman H, Alenius H, Laitinen A. Inflammation and functional outcome in diisocyanate-induced asthma after cessation of exposure. *Allergy* 2008;63:583-591
- III) **Meuronen A**, Karisola P, Leino M, Savinko T, Sirola K, Majuri ML, Piirilä P, Virtanen I, Mäkelä M, Laitinen A, Laitinen LA, Alenius H. Attenuated expression of tenascin-C in ovalbumin-challenged STAT4^{-/-} mice. *Respir Res.* 2011;12:2
- IV) **Meuronen A**, Laitinen A, Altraja A, Virtanen I, Halme M, Nieminen EM, Kava T, Haahtela T, Sovijärvi A, Lindqvist A, Laitinen LA. Expression of laminin chain $\alpha 2$ in the bronchial basement membrane may contribute to the development of asthma. Submitted.

The publications are referred to in the text by their Roman numerals. The original publications are reprinted with the permission of the copyright holders. In addition, some unpublished results are presented.

Abbreviations

| | |
|-------|--|
| AHR | airway hyperreactivity |
| APAAP | alkaline phosphatase anti-alkaline phosphatase |
| APC | antigen presenting cell |
| ASM | airway smooth muscle |
| BAL | bronchoalveolar lavage |
| BALF | BAL fluid |
| BM | basement membrane |
| CCL | CC chemokine ligand |
| CCR | chemokine receptor |
| CD | cluster of differentiation |
| cDNA | complementary DNA |
| COPD | chronic obstructive pulmonary disease |
| CS | cigarette smoke |
| Ct | threshold cycle |
| CXCL | CXC chemokine ligand |
| DC | dendritic cell |
| DI | diisocyanate |
| DIA | diisocyanate-induced asthma |
| DNA | deoxyribonucleic acid |
| ECM | extracellular matrix |
| ECP | eosinophil cationic protein |
| EDN | eosinophil derived neurotoxin |
| EGF | epidermal growth factor |
| EMTU | epithelial-mesenchymal trophic unit |
| EPO | eosinophil peroxidase |
| EPX | eosinophil protein X |
| FEV1 | forced expiratory volume in 1 s |
| FN | fibronectin |
| FVC | forced vital capacity |
| GR | glucocorticoid receptor |
| HDI | hexamethylene DI |
| i.p. | intraperitoneal |
| ICS | inhaled corticosteroids |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| KO | gene knockout |
| LPS | lipopolysaccharide |
| LT | leukotriene |
| MBP | major basic protein |
| Mch | metacholine |
| MDI | diphenylmethane DI |

| | |
|--------|---|
| MIP | macrophage inflammatory protein |
| MMP | matrix metalloproteinase |
| MPO | neutrophil myeloperoxidase |
| NK | natural killer |
| NSBHR | non-specific bronchial hyperreactivity |
| OVA | ovalbumin |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEF | peak expiratory flow |
| Penh | enhanced pause |
| PG | prostaglandin |
| RANTES | regulated on activation, normal T-cell expressed and secreted |
| RNA | ribonucleic acid |
| RT | room temperature |
| RT-PCR | reverse transcription PCR |
| RU | relative expression unit |
| SD | standard deviation |
| SEM | standard error of mean |
| STAT | signal transducer and activator of transcription |
| TDI | toluene DI |
| TGF | transforming growth factor |
| Th | T-helper |
| TNF | tumour necrosis factor |
| Treg | regulatory T cells |
| TSLP | thymic stromal lymphopoietin |
| WBP | whole-body plethysmography |
| WT | wild-type |

Abstract

Asthma is a chronic inflammatory disorder of the airways. Remodelling in asthma is defined as the structural changes seen in the airways of asthmatics in comparison to healthy controls. Progressive loss of lung function also seen in asthma might be caused by remodelling. The research aims of this thesis were to investigate inflammation and remodelling in the airways of different types of asthmatics and smokers. The association between inflammation and remodelling was also examined in a mouse model of allergic airway inflammation.

Bronchoscopy and lung function tests were performed on patients with newly diagnosed asthma, with asthma symptoms but no asthma diagnosis, with diisocyanate-induced asthma (DIA), and on smokers and control subjects. Biopsies and bronchoalveolar (BAL) samples were collected. Inflammatory cell numbers were quantified in the biopsies and the BAL samples from smoking and non-smoking controls and patients with DIA, and tenascin-C immunoreactivity was measured in the bronchial basement membrane (BM). mRNA expression of a wide variety of inflammatory mediators was assessed in BAL cells with relative quantitative real-time RT-PCR. DIA patients were followed-up for 2–3 years and they were on inhaled corticosteroid (ICS) medication. Laminin expression was examined by semiquantitative immunohistochemistry in the BM of patients with newly diagnosed asthma, those with asthma symptoms and non-smoking controls. Tenascin-C expression in allergic airway inflammation was studied in wildtype (WT), STAT4^{-/-} and STAT6^{-/-} mice that were sensitised and challenged with OVA or PBS (controls). The expression of mRNA for inflammatory mediators, laminin and tenascin-C was investigated in the lung. Laminin and tenascin-C protein expression was evaluated with immunohistochemistry. The results of mouse model studies were confirmed *in vitro* with human fibroblasts.

Healthy smokers showed increased numbers of macrophages in the BAL with no changes in the inflammatory cells in biopsies. Macrophages seemed to be quite quiescent, since mRNA expression for a wide variety of inflammatory mediators, especially chemokines CCL3, CCL4, CCL5 and CCL20, secreted by macrophages was significantly lower than in healthy non-smokers. Attenuated macrophage activity in the airway lumen may render smokers more susceptible to airway infections and have an impact on the development of other airway pathology.

DIA patients had elevated numbers of IL-4-producing cells in the lamina propria at the beginning compared to controls, probably as a sign of Th2-type inflammation, but the difference disappeared during the follow up. Patients with DIA on ICS who still had non-specific bronchial hyperreactivity (NSBHR) at the end of the follow-up showed increased expression of TNF- α , IL-6 and IL-15 mRNA in BAL cells compared to those without NSBHR. In addition to being markers for poor prognosis and possible slight glucocorticoid

resistance, these cytokines might aid in guiding the treatment of DIA. DIA patients had only a slight but significant increase in the thickness of the tenascin-C immunoreactive layer under the epithelium without correlation with NSBHR. The increase in the thickness was much less than usually seen in other types of asthma, which might not make tenascin-C a good marker for DIA, and probably reflects different mechanisms in disease pathogenesis in comparison to classic allergic asthma.

OVA-induced tenascin-C expression in the lung was attenuated in STAT4^{-/-} mice with impaired Th1-type immunity compared to WT mice. Interestingly, STAT6^{-/-} mice with impaired Th2-type immunity showed tenascin-C expression levels similar to those of WT mice. The clearest difference between these two knockout strains in response to OVA was that STAT4^{-/-} mice exhibited no upregulation of IFN- γ and TNF- α mRNA expression. Thus, tenascin-C expression was unexpectedly more related to Th1 type reactions. *In vitro* studies confirmed the results. Human fibroblasts stimulated by TNF- α and IFN- γ showed increased expression of tenascin-C.

Patients with newly diagnosed asthma showed increased expression of laminin $\alpha 2$ in comparison to patients with asthma symptoms only and healthy controls. Both patients with asthma and those with only asthma symptoms showed increased expression of the laminin $\beta 2$ chain in comparison to controls. Thus, laminin $\alpha 2$ expression differentiated patients with clinical asthma from patients with symptoms only. Furthermore, the expression of laminin $\alpha 2$ and $\beta 2$ was associated with NSBHR, linking very specific remodelling events to clinical disease.

1. Review of the literature

1.1 *Inflammation in the airways*

1.1.1 Inflammatory mediators

Inflammatory reactions in the airways are orchestrated by inflammatory and stationary cells as well as inflammatory mediators secreted by them. Study of the basic actions of these mediators in animal models and cell cultures has aided in understanding inflammatory diseases of the lungs (Chung 2001, Commins et al. 2010, Holgate 2008). Cytokines are small secreted cell-signalling proteins with paracrine, autocrine and even endocrine functions. Chemokines are an important subgroup of cytokines and are here discussed separately. Chemokines have been named for their ability to attract cells, a phenomenon called chemotaxis. Cytokines and chemokines are essential mediators of inflammation and immunity and are produced and secreted in response to various stimuli (Chung 2001, Commins et al. 2010). The original names for cytokines and chemokines derive from their history. Interleukins (IL) are typical cytokines. Their name points to the connection between leukocytes, which were the first cells to be discovered to secrete ILs. Moreover, the name tumour necrosis factor (TNF) indicates how the TNF superfamily was discovered. Cytokines and chemokines can be categorised in many ways. Traditionally, they are classified into proinflammatory cytokines, Th (T-helper) 1, Th2 and Th17 cytokines, and chemokines according to their function and the cells they are secreted by (Borish and Steinke 2003, Commins et al. 2010, Dinarello 2000). The numbers of known cytokines and chemokines have constantly been growing. The categorisations are artificial and simply intended to help in understanding the complex network of inflammatory mediators. Cytokines and chemokines are also secreted by many other cell types than inflammatory cells. The field is so vast that only the cytokines important for this thesis are covered.

Cytokines

The proinflammatory cytokines, such as TNF- α , IL-1 and IL-6, promote inflammation by activating a variety of inflammatory mediators that are crucial in the cascade of events leading to tissue inflammation, even directly promoting matrix metalloproteinase (MMP) release (Borish and Steinke 2003, Chung 2001). They are mostly secreted by macrophages and other antigen presenting cells (APC), although many other cells can also secrete them. Proinflammatory cytokines are produced and released in response to various stimuli from bacterial products to other cytokines. TNF- α has numerous effects, such as an antitumor capacity, and it is an important mediator in innate immunity. (Berry et al. 2007, Chung 2001, Commins et al. 2010). It has direct cytotoxic effects and induces further immune responses. It is the primary mediator in septic shock and chronic inflammation-related cachexia. TNF- α

stimulates the expression of other proinflammatory mediators, leukocyte adhesion factors, and CCL (CC chemokine ligand) 5, among others. TNF- α inhibitors are mainly used in the treatment of rheumatoid arthritis and other autoimmune diseases (Sfikakis 2010). They have additionally been tested in the treatment of asthma and COPD, mainly with negative results (Matera et al. 2010). However, some patients with severe asthma have been reported to benefit from the inhibition of TNF- α (Berry et al. 2007, Matera et al. 2010). IL-1 and TNF- α are often released together and they have similar actions making it sometimes difficult to separate the functions of these cytokines from each other (Commins et al. 2010, Dinarello 2000). In addition to activities in common with TNF- α , IL-1 stimulates T-lymphocytes similarly to IL-6. The latter is secreted by various cells, including macrophages and epithelial cells, and has multiple functions, many of which are quite similar to TNF- α and IL-1. IL-6 stimulates acute phase protein synthesis, induces fever, promotes a Th1-type immune response and also inhibits IL-1 and TNF- α production (Borish and Steinke 2003, Dodge et al. 2003, Jirapongsananuruk et al. 2000, Yu et al. 2002).

Th1 and Th2 cytokines promote cell-mediated immunity and allergic responses, respectively. They are named after the subsets of CD (cluster of differentiation) 4+ Th cells secreting them, although they are also produced by other cells (Borish and Steinke 2003). Classic Th1 cytokines include IFN- γ and TNF- β , among others, while IL-4, IL-5 and IL-13 are the primary Th2 cytokines. New members of the latter include IL-9, IL-23 and IL-31.

IL-12 is a primary cytokine driving Th1 cell polarisation. It induces the expression of interferon (IFN)- γ , which is a major **Th1 cytokine** primarily secreted by T cells upon antigen challenge, natural killer (NK) cells and macrophages. It activates macrophages, dendritic cells, NK cells and neutrophils (Antoniou et al. 2003, Commins et al. 2010). Thus, IFN- γ is very important in combating intracellular pathogens. It inhibits collagen synthesis and has been proposed as a treatment for patients with diseases manifesting as fibrosis (Antoniou et al. 2003). IL-15 is classified in this review with the Th1 cytokines because of its functions, although it is mainly secreted by APC cells, similarly to proinflammatory cytokines. Most importantly, IL-15 activates NK cells. It is also a growth factor of T-lymphocytes and, to a lesser extent, mast cells. IL-15 is responsible for the survival of CD8+ memory cells and it attracts T cells and neutrophils (Commins et al. 2010, D'Acquisto et al. 2010).

Th2 cytokines IL-4, IL-5 and IL-13 are able to induce most of the hallmarks of atopic asthma: mucus overproduction, goblet cell hyperplasia, non-specific bronchial hyperreactivity (NSBHR; synonyms are airway hyperreactivity, AHR, or bronchial hyperreactivity, BHR) and eosinophilia (Holgate 2008, Paul and Zhu 2010). NSBHR is measured as a reduction in airway flow in response to increasing concentrations of histamine or metacholine (Sovijarvi et al. 1993) and is a central feature of asthma. IL-4 is the most important cytokine in the initiation of Th2-type responses. It induces Th0 lymphocytes to differentiate into Th2 cells

and down-regulates the production of proinflammatory cytokines. Eosinophils are the main target for IL-5, which is involved in almost every aspect of eosinophil proliferation, maturation and activity (Commins et al. 2010). As elevated numbers of eosinophils are a key feature of asthma (Holgate 2008), inhibition of the action of IL-5 was considered to be an obvious choice for the treatment of asthma (Foster et al. 1996). Despite a fall in circulating, sputum and airway eosinophil counts, the results of human trials were disappointing, with only patients suffering from severe eosinophilic asthma with exacerbations having some benefit from blocking the actions of IL-5 (Flood-Page et al. 2003b, Haldar et al. 2009, Nair et al. 2009). This outcome weakened the suspected role of eosinophils in asthma and highlighted the diversity of the disease (*see* also the section “Allergic asthma and mouse models of airway inflammation” for eosinophil). IL-13, referred to as the effector in Th2-type adaptive immunity, is important in the defence against parasites and a well-characterised and widely expressed mediator of allergic inflammation and tissue fibrosis (Commins et al. 2010, Wynn 2003).

Chemokines

Chemokines have a wide variety of actions in inflammation, mostly related to the chemotaxis of inflammatory cells (Borish and Steinke 2003, Commins et al. 2010, Conti and DiGioacchino 2001). For clarity, the chemokines referred to in this thesis with their nomenclature are listed in Table 1. Systematic names were introduced because the same chemokine might have been discovered in different instances and thus had several common names. They also participate in guiding T-cell differentiation and tissue homeostasis. Chemokines can be divided according to the position of cysteine residues in the N-terminal into two main groups that comprise almost all chemokines: CXC (α -chemokines) and CC (β -chemokines). Those included in the former group chiefly attract neutrophils, while mediators in the latter group attract the other inflammatory cells. X stands for a variable amino acid. Other small groups include C- and CX3C-chemokines. CCL3, CCL4 and CCL20 are macrophage inflammatory proteins (MIPs) that belong to the CC chemokines and are produced by activated macrophages and other inflammatory cells (Commins et al. 2010, Driscoll 1994, Maurer and von Stebut 2004). In addition, many other cells, including peripheral blood leukocytes and vascular smooth muscle cells, are capable of producing MIPs under stimulation. CCL3 and CCL4 attract activated T-cells, monocytes, dendritic cells and NK cells bearing chemokine receptor 1 (CCR1) and CCR5 on their surface, as well as CCL3- and CCR1-positive cells. CC chemokines functioning through CCR5 can stimulate the development of IFN- γ -producing Th1 cells (Commins et al. 2010). CCL20 recruits CCR6-positive dendritic cells and possesses an antimicrobial capability (Yang et al. 2003). Regulated on activation, normal T-cell expressed and secreted RANTES, also known as CCL5, is a CC chemokine expressed in various cells including fibroblasts, macrophages and epithelial cells (Berkman et al. 1996, Casola et al. 2002, Chung 2001). It mainly recruits eosinophils and basophils, and to lesser extent T-lymphocytes. Eotaxin/CCL11 is a potent

eosinophil chemoattractant. Immunoglobulin (Ig) E receptor crosslinkage on the mast cell surface translocates CCR3 to the cell surface, and binding of the CCL11 to this CCR3 potentiates IgE-dependent IL-13 production (Price et al. 2003). CCL11 is released by roughly the same cells as CLL5 (Commins et al. 2010). Asthmatics have more CCL11-positive fibroblasts in bronchial biopsies than controls (Wenzel et al. 2002). Many cells possess the ability to release the powerful neutrophil chemoattractant and activator CXCL8 in response to various stimuli, the most important cells in the airways probably being macrophages and epithelial cells (Commins et al. 2010). Expression of CXC-chemokine ligand (CXCL) 8 is especially stimulated by bacterial products, TNF- α , IL-1 and viral infections. It induces neutrophil adherence to endothelial cells and respiratory burst.

Table 1. Nomenclature for chemokines referred to in this thesis

| <i>Systematic name</i> | <i>Common name(s)</i> | <i>Receptor(s)</i> |
|------------------------|-------------------------------|------------------------|
| CCL3 | MIP-1 α /LD78 α | CCR1, CCR5 |
| CCL4 | MIP-1 β | CCR5 |
| CCL5 | RANTES | CCR1, CCR3, CCR4, CCR5 |
| CCL11 | Eotaxin | CCR3 |
| CCL20 | MIP-3 α , LARC | CCR6 |
| CCL22 | MDC, STCP-1 | CCR4 |
| CCL24 | Eotaxin-2, MPIF-2 | CCR3 |
| CCL26 | Eotaxin-3 | CCR3 |
| CXCL8 | IL-8 | CXCR1, CXCR2 |

Adapted from Commins et al. (2010)

1.1.2 The role of smoking in airway inflammation

Among the many other detrimental consequences, cigarette smoke has a major impact on inflammation in the airways (Amin et al. 2003, Aoshiba et al. 2001, Barbers et al. 1987, Barnes 2010, Di Stefano et al. 1998, Dippolito et al. 2001, Ekberg-Jansson et al. 2000, Hagiwara et al. 2001, Kotani et al. 2001, Lams et al. 1998, Lams et al. 2000, Linden et al. 1993, Mio et al. 1997, Takizawa et al. 2000). Smoking is associated with a longer duration of respiratory infections (Huttunen et al. 2010) and a high incidence of chronic obstructive pulmonary disease (COPD) (Eisner et al. 2010). Depending on the diagnostic criteria, smoking status and age, 12–50% of smokers develop COPD, but 60–80% of COPD cases are evaluated to be attributed to smoking (Eisner et al. 2010, Lundback et al. 2003, Weiss et al. 2003). As with other multifactorial diseases, it is far from clear why some smokers develop COPD while others do not, although much has been discovered about the genetic background predisposing to COPD (Molfino 2004).

COPD is an immunological and inflammatory disease of the smaller airways with systemic manifestations (Cosio and Agusti 2010). In COPD the limitation of airway flow is partly

irreversible, in contrast to asthma, although elderly people often have a double diagnosis (Gibson and Simpson 2009). Although the inflammation in asthma and COPD is not similar, there might be common factors for susceptibility to both. For instance, IL-13 promoter polymorphisms have been linked to both COPD and asthma (Molfino 2004). Furthermore, NSBHR and a family history of asthma are risk factors for COPD (de Marco et al. 2010). Patients with COPD seem to be as heterogeneous a population as those with asthma (Garcia-Aymerich et al. 2010). Depending of the severity and stability of the disease, macrophages, CD8+ cells and neutrophils are variably deposited in the airway wall and lumen in the upper and lower parts of the respiratory tract (Barnes 2000, Jeffery 2004, Saetta 1999). In COPD the expression of proinflammatory cytokines and chemokines such as IL-6, IL-1 β , TNF- α , CXCL8, CCL2 and CCL5 is elevated in the airways (Chung 2001).

In asymptomatic smokers, inflammation in the airways is not as well defined. A consistent finding in bronchoalveolar lavage (BAL) samples from asymptomatic smokers is elevated numbers of macrophages compared to non-smokers (Barbers et al. 1987, Hagiwara et al. 2001, Kotani et al. 2000, Kotani et al. 2001, Linden et al. 1993). However, smoking suppresses macrophage function against pathogens in asymptomatic smokers (Kotani et al. 2000, Kotani et al. 2001, McCrea et al. 1994, Ohta et al. 1998, Yamaguchi et al. 1993). Alveolar macrophages participate in recognizing foreign antigens as pathogens and they secrete a variety of cytokines and chemokines, which are involved in the initiation and maintenance of inflammatory responses (Borish and Steinke 2003, Driscoll 1994, Gordon and Read 2002). They phagocytise foreign material as well as release reactive oxygen species, antibacterial products and leukotrienes. Healthy smokers have shown ineffective alveolar macrophage function compared to non-smokers measured by the response to LPS: lower TNF- α (McCrea et al. 1994, Yamaguchi et al. 1993), IL-6 (McCrea et al. 1994), IL-1 β (Sauty et al. 1994) and CXCL8 release (Ohta et al. 1998). Consistently with this, smokers in previous studies have exhibited increased macrophage aggregation but decreased IL-1 β , IFN- γ , and TNF- α mRNA expression and lowered phagocytic as well as microbicidal activity during anaesthesia compared to non-smokers, even if patients stop smoking six months before operation (Kotani et al. 2000, Kotani et al. 2001). There have been few biopsy studies comparing inflammatory cell numbers in asymptomatic smokers and non-smokers without any other pulmonary pathology (Amin et al. 2003, Ekberg-Jansson et al. 2000).

Likewise, the cytokine and chemokine profile in the BAL fluid (BALF) of asymptomatic smokers is not very clear (Hagiwara et al. 2001, Kotani et al. 2000, McCrea et al. 1994, Ohta et al. 1998, Takizawa et al. 2000, Tanino et al. 2002). Only a few mediators have been studied. Even more heterogeneous have been the *in vitro* results with cigarette smoke extract exposure, as reported in a recent review by Smith et al. (2010).

1.1.3 Allergic asthma and mouse models of allergic airway inflammation

Asthma is a very common disease with a global burden affecting an estimated 300 million people worldwide. According to the definition of the Global Initiative for Asthma (GINA), asthma is “a chronic inflammatory disorder of the airways” and “the chronic inflammation is associated with airway hyperresponsiveness” leading to asthma symptoms (GINA. 2009). Thus, airways become obstructed under suitable conditions and the obstruction is usually reversible. This is of course a very broad and descriptive statement. In everyday life, diagnosis is mainly based on symptoms and the verification of airflow limitation in lung function tests such as spirometry, peak expiratory flow (PEF) follow-up, and in some cases by determining NSBHR. Symptoms include a history of cough (at night), chest tightness, wheezing and difficulties in breathing. Spirometry aims at confirming the reversibility of the obstruction. According to the GINA guidelines, the diagnostic criteria for asthma include a post-bronchodilator increase in the forced expiratory volume in 1 s (FEV1) $\geq 12\%$ and at least 200 ml. In PEF follow-up, diurnal variation over 20% is regarded as diagnostic when recurring in 10% of measurements or a bronchodilator response $\geq 20\%$ / 60 l/min. As the diversity of the disease has become evident, guidelines handling asthma as one disease with different degrees of severity have not always been adequate (Hargreave and Nair 2009). As with other common multifactorial diseases, there are many environmental risk factors acting on certain genetic backgrounds leading to various phenotypes (Meyers 2010). These phenotypes present with the same symptoms and clinical findings. This is problematic when modelling the disease. Currently, routine treatment roughly relies on suppressing the inflammation (glucocorticoids, leukotriene inhibitors as additional medication) as well as relieving bronchoconstriction (β_2 agonists, anticholinergics). Glucocorticoids suppress inflammation very widely, which explains their efficacy in different types of asthma and other inflammatory diseases (Barnes 2010). In addition to the main effects, every drug has other functions in the airways (Mauad et al. 2007). The relationship between symptoms and inflammatory markers is not consistent (Tillie-Leblond et al. 2009). Symptom-based guidance of treatment may predispose to an increased number of exacerbations. Thus, patients might benefit from inflammatory marker-directed therapy (Tillie-Leblond et al. 2009).

Although it is even more artificial to divide asthma into allergic (or atopic) and non-allergic forms based on the atopic history or IgE production in clinic, this section will focus on typical asthmatic airway inflammation most strongly associated with allergic asthma. The ovalbumin (OVA)-induced mouse model of asthma used in this thesis simulates eosinophilic, IgE-dependent airway inflammation leading to NSBHR.

Airway inflammation in (allergic) asthma

In humans, asthmatic airway inflammation is governed by an increased number of eosinophils, mast cells, CD4+ lymphocytes, neutrophils, macrophages and cytokines of Th2-type inflammation (Holgate 2008). The inflammation covers the whole airway from allergic rhinitis to mild asthma in the bronchial mucosa always down to the small airways and inflammation in the submucosa and muscle in more severe asthma. All the real inflammatory cells are derived from bone marrow.

Eosinophils are considered to have a key role in allergic inflammation. They are circulatory cells that are activated by various stimuli ranging from complement and tissue injury to cytokines. Their well-known ability in innate immunity is to chemically attack the body's own tissues as well as parasites by releasing the components of their granules: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN, also known as eosinophil protein X, EPX). In addition, eosinophils secrete a wide variety of mediators capable of further inducing inflammation and tissue fibrosis, including leukotrienes (LT), transforming growth factor- β (TGF- β), granulocyte macrophage colony-stimulating factor (GM-CSF), neuromediators and the classic Th2 immune response-related cytokines and chemokines (IL-4, IL-5, IL-13, CCL5, CCL11), among others. Moreover, they have the ability to act as APCs, increase the reactivity of smooth muscle and activate mast cells (Rothenberg and Hogan 2006). Thus, eosinophils may influence Th1/Th2 polarization and NSBHR, underlining their importance in the Th2-type inflammatory response and contribution to many of the features of asthma. The only slight effect of IL-5 antibodies on asthma symptoms and clinical parameters has also been attributed to the fact that the inhibition of eosinophil activity and recruitment is only 50% in biopsies (*see* also the section "Inflammatory mediators – Cytokines") (Flood-Page et al. 2003b, Holgate 2008). Eosinophils are also recruited by other mediators such as CCL5 and CCL11, CCL24 and CCL26 (Rothenberg and Hogan 2006). Although IL-5 antibodies had little effect on asthma symptoms, remodelling of the airways was reduced when measured in terms of the expression of tenascin, lumican and procollagen III (Flood-Page et al. 2003a), which further associates eosinophilic airway inflammation with remodelling. It is notable that an excess of eosinophils in the airways does not necessarily lead to NSBHR (eosinophilic bronchitis), and NSBHR can exist without eosinophils (Bradding et al. 2006, Kim et al. 2010b, Rothenberg and Hogan 2006)

Mast cells are another prominent and important cell type in asthma. The total numbers are not necessarily increased, but the activation and localisation of mast cells seems to be more important, i.e. their presence in deep/superficial or upper/lower parts of the airways (Bradding et al. 2006). Mast cells are stationary cells in tissues and are best known as mediators of allergy. When an IgE-primed mast cell meets an allergen, IgE receptors crosslink and the mast cell releases its granules into the tissue, as well as secreting *de novo* mediators. Other

stimuli such as complement, viral antigen or tissue injury can also activate degranulation and mast cells (Bradding et al. 2006, Okayama et al. 2007, Theoharides and Kalogeromitros 2006). In asthma, mast cells are constantly active. Histamine, LTs and prostaglandin (PG) D₂ released by mast cells are important effectors in asthma pathology, since they can cause bronchoconstriction, mucosal oedema, and mucus secretion. In addition, mast cells can secrete a wide diversity of substances, many with importance in asthma, including cytokines (also IL-4, IL-5, and IL-13), chemokines (also CCL5), peptides, proteoglycans, enzymes (also MMPs) and growth factors. Mast cells can be divided into two groups: mucosal and connective tissue types (Bradding et al. 2006, Holgate 2008, Okayama et al. 2007). Mucosal mast cells function under the guidance of T-lymphocytes. Deeper connective tissue mast cells are more dependent on stem cell factor (SCF), which is upregulated in asthma. More research attention has been focused on the deep connective tissue mast cells (Bradding et al. 2006). These are situated in close connection with smooth muscle and may interact with smooth muscle cells. Thus, mast cells also provide a connection between airway inflammation and remodelling, although with little evidence in humans.

T-lymphocytes have long been at the centre of attention in asthma research. Enormous progress has been made during the last decade in understanding the role of T cells in asthma and other diseases (Lloyd and Hessel 2010). As T cell research has advanced, the number of different subgroups has grown and all may have a role in asthma. Different T-cell subtypes and phenomena related to their action and asthma are listed in Table 2, with the main cytokines they secrete. Asthma is mainly characterised by increased numbers of CD4⁺ lymphocytes, also known as Th cells, which have been thought to be of the Th2 subtype. However, it is probably not this simple, as demonstrated in Table 2. Moreover, cells have been also recognised in the lungs capable of secreting IL-13 and IL-4 under cytokine stimulation, i.e. from the epithelium, without adaptive immunity and T cell receptors (TCR) (Lloyd and Hessel 2010). These are innate immune cells that do not bear markers of any known leukocyte lineage (Neill et al. 2010, Price et al. 2010). It was previously thought that when CD4⁺ cells commit, they are no longer able to change their secretion of cytokines, but this has also been questioned, as Th2 cells were shown to be able to transform into IL-9 producing cells (Lloyd and Hessel 2010, Veldhoen et al. 2008). TGF- β seems to have a key role (Veldhoen et al. 2008).

Although allergic asthma has been considered an eosinophilic phenomenon without increased numbers of **neutrophils** in the stable phase, neutrophils are often found in patients with severe and glucocorticoid-unresponsive asthma as well as in relation to exacerbations (Barnes 2007). Neutrophils are among the first cells at the site of inflammation and are active phagocytes. They secrete substances such as elastase, thromboxane A₂, MPO, MMPs and LTB₄, directly causing features of asthma including bronchoconstriction, oedema and mucus hypersecretion, and possibly participating in remodelling (Monteseirin 2009).

Neutrophils also increase chemotaxis and the activation of other cells important in asthma by secreting cytokines and chemokines. New interest in neutrophils has also been raised by neutrophil recruiter IL-17, a cytokine produced by a subset of CD4+ lymphocytes designated as Th17cells (Alcorn et al. 2010, Harrington et al. 2005, Park et al. 2005b). To a lesser extent, neutrophils, eosinophils, $\gamma\delta$ cells and CD8+ T cells are able to secrete IL-17. Its expression is increased in asthmatics, although the cellular source is not entirely clear. More interestingly, IL-17 might be more related to allergic than non-allergic asthma (Alcorn et al. 2010, Wang et al. 2010).

Table 2. T cell subtypes, cytokines secreted by them and action in asthma models

| <i>T cell subtype</i> | <i>Cytokine(s)</i> | <i>Actions</i> |
|-----------------------|--------------------------------|--|
| Th1 | IFN- γ | <ul style="list-style-type: none"> Unclear; role in exacerbations? |
| Th2 | IL-4 IL-5 IL-13 | <ul style="list-style-type: none"> Th2 differentiation Mast cell recruitment Eosinophilia IgE class switching Mucus production NSBHR |
| Th9 | IL-9 | <ul style="list-style-type: none"> Mast cell recruitment IgE class switching Mucus production |
| Th17 | IL-17 | <ul style="list-style-type: none"> Chemokine production Neutrophilia Role in glucocorticoid resistant asthma? |
| Treg | IL-10 | <ul style="list-style-type: none"> Suppression of inflammatory responses (both adaptive and innate) |
| CD8+ | IFN- γ IL-4 IL-13 | <ul style="list-style-type: none"> Correlates with disease severity Eosinophilia NSBHR Role in viral exacerbations |
| NKT | IFN- γ IL-13 | <ul style="list-style-type: none"> Eosinophilia IgE class switching NSBHR |
| $\gamma\delta$ | IL-4 IL-5 IL-13 IL-17 | <ul style="list-style-type: none"> Vγ1 subtype: proinflammatory, secrete IL-5 and 13, promote NSBHR Vγ4: suppressors, produce IL-17A, suppress NSBHR |

Adapted from Lloyd and Hessel (2010).

The number of **macrophages** is often elevated in the airway mucosa in asthma, but rather little is known about their eventual role in the pathogenesis of allergic asthma. However, the role of macrophages and especially dendritic cells has begun to be clarified during the past few years, as they have also been shown to be able to polarize towards driving the Th2-type immune response (Lambrecht and Hammad 2010). Traditionally, they are seen as promoters of Th1-type immunity. Macrophages and dendritic cells differentiate from monocytes under the influence of GM-CSF. They serve as APCs (dendritic cells), phagocytes (macrophages), an important source for cytokines and other mediators, and are considered the key cell

between innate and adaptive immunity (Gordon and Read 2002). Antigen presentation to lymphocytes is a crucial step in the development and activation of adaptive immunity. Macrophages are thought to have a role in glucocorticoid-resistant asthma (Barnes and Adcock 2009), severe asthma in children (Fitzpatrick et al. 2011) and in animal models of chronic Th2-type inflammation and NSBHR (Byers and Holtzman 2010, Yang et al. 2010).

The number of **basophils**, circulating effectors of IgE-mediated allergic reactions, is also elevated in biopsies from asthmatic subjects (Macfarlane et al. 2000). As with mast cells, IgE receptor-mediated triggering is not the only way to activate basophils, and they also have the ability to function as APCs driving Th2 polarisation (Yoshimoto 2010). The role of basophils in asthma is not clear.

The airway epithelium and other stationary cells also have implications in inflammation, as they are affected by inflammatory cells and mediators in the airways. Stationary cells secrete inflammatory mediators and can respond directly to external stimuli. The epithelium is the barrier between the lumen and tissue in the airways and thus has an important role in the defence against external threats. According to one view, in susceptible individuals under suitable environmental conditions, this might be not as effective and well directed, resulting in the progression of asthma (Holgate 2008, Lambrecht and Hammad 2010, Lloyd and Hessel 2010) *see* the section on remodelling.

Immunity

For many years after the discovery of inflammation in the airways, asthma was considered almost solely a disease of disturbed adaptive immunity, while in recent years the role of innate immunity has also become evident (Bonilla and Oettgen 2010, Holt and Strickland 2010, Turvey and Broide 2010). Innate immunity is the reaction of organisms to threats they are facing for the first time. These responses are nonspecific, ranging from enzymes in saliva to pattern recognition by cells involved in innate immunity. The cells participating include granulocytes (eosinophils, neutrophils and basophils), NK cells (cytotoxic lymphocytes distinct from NKT cells) and cells of the monocyte lineage such as macrophages. Depending on the point of view, the epithelium and other barriers and stationary cells may sometimes be categorised as components of innate immunity, although the role of the epithelium is much more besides that (Lambrecht and Hammad 2010).

Cells involved in innate immunity also phagocytose material and present it to T-lymphocytes in lymph nodes, thus linking innate and adaptive immunity. In adaptive immunity, APCs (macrophages, dendritic cells, B-cells and some epithelial cells) present antigens received from the digestion and processing of foreign material in major histocompatibility class (MHC) II to Th cells in the presence of co-stimulatory signals in lymph cell accumulations (Bonilla and Oettgen 2010). These co-stimulatory signals define how strong a response is

elicited. The APC and cytokine milieu defines the orientation of Th cells. T cells recognise antigens with their TCR. T cells proliferate and are ready to elicit a much greater response on the next counter. CD8⁺ cells recognise antigens bound to MHC I molecules and are important in recognising the body's own tissues and intracellular pathogens. B-cells have Ig on their surface which functions as a B-cell receptor (BCR). Antigen binds to this and with a co-stimulatory signal from Th cells activates B cells, which proliferate and begin producing antibodies, establishing a new antigen-specific clone. Under the influence of IL-4, B cells undergo class switching, changing the type of Ig they produce to IgE. Both T cells and B cells undergo selection during development in order to prevent immune reactions that are too strong or wrongly guided. In normal situations, tolerance towards inhaled antigens should develop, but something goes wrong in asthma (Holt and Strickland 2010). Both B and T cells have effector and memory cells. This is, of course, a very simplified view nowadays.

Allergen challenge in the airways

In allergen challenge, the above machinery is orchestrated to produce allergic inflammation. When an allergen and cells with allergen-specific IgE and dendritic cells meet, rapid activation of macrophages and mast cells follows (Bousquet et al. 2000, Holgate 2008, Paul and Zhu 2010). These activated cells provoke immediate allergic inflammation, as discussed above, resulting in contraction of ASM, vasodilation, exudation of plasma into the airway wall and lumen, and mucous overproduction. This is a simplified view of the early-phase reaction that occurs immediately. In the late-phase reaction, over 6 h after the challenge, CD4⁺ cells, neutrophils, basophils and eosinophils join the reaction with their specific actions, as above, recruited by mast cells and macrophages. Dendritic cells that have travelled to lymph cell collections present the allergen as antigens. The importance of eosinophils and basophils as APCs is not very clear *in vivo*. In this phase, a Th2-type inflammatory mediator pattern may already be seen, but is probably dependent on other cells than Th2 cells. The infiltration of cells also requires an increase in endothelial adhesion molecules for inflammatory cells. After the first 24 hours, activated T cells are seen, resulting in an adaptive immune response by the previously sensitised immune system.

Animal models

Animal models of allergic inflammation should mimic the features of human asthma and preferably represent remodelling under chronic exposure. Furthermore, the development of an allergic response should be similar with sensitisation and challenge to that in humans. A late-phase reaction should be seen. On the other hand, most of the data on the mechanisms of inflammation in asthma are from animal studies (Kips and Pauwels 1999). Differences are apparent between species, and even mouse strains, in the way they react to antigen sensitisation and challenge (Hylkema et al. 2002, Kirschvink and Reinhold 2008). Mice are probably not the best model species, but are quite close to humans, are an economical species for research, and numerous genetically manipulated strains are available (Bates et

al. 2009, Ricciardolo et al. 2008). Agents used to elicit an allergic response should also have similarities with human allergens, but then again mice have no spontaneous asthma. Balb/c mice are born with a strong tendency for atopy. They are highly reactive to ovalbumin (OVA) and present remodelling changes after prolonged challenge, in addition to Th2-type eosinophilic inflammation, NSBHR, goblet cell hyperplasia and elevated plasma levels of IgE (Kumar and Foster 2002). Even in OVA sensitised and challenged mice, the dose, duration, adjuvants, route of sensitisation and challenge, and the time of sacrifice, among other factors, affect the result (Kumar et al. 2008). Variation might occur within the same strain with the same experimental protocol between mice from different suppliers and between sexes (Allen et al. 2009). The model should be chosen according to the phenomenon intended to be studied (Zosky and Sly 2007). Below is a list (Table 3) of the best known animal models of asthma.

Table 3. Animal models of asthma.

| <i>Species</i> | <i>Antigen</i> | <i>Reference</i> |
|-------------------|----------------------------|---|
| Mouse | OVA | Renz et al. 1992 |
| | House dust mite | Johnson et al. 2004 |
| | Cockroach antigen | Lundy et al. 2003 |
| | Aspergillus fumigatus | Kurup et al. 1992 |
| | Ragweed | Sur et al. 1996 |
| | Sheep red blood cells | Gavett et al. 1994 |
| Rat | Hexamethylene diisocyanate | Herrick et al. 2002 |
| | OVA | Hylkema et al. 2002 |
| | House dust mite | Singh et al. 2003 |
| Guinea pig | Ascaris | Misawa et al. 1987 |
| | OVA | Thorne and Karol 1989 |
| Dog | Toluene diisocyanate | Karol 1987 |
| | Ascaris | Booth et al. 1970, Hirshman et al. 1980 |
| | Ragweed | Redman et al. 2001 |
| Sheep | Ascaris | Abraham et al. 1988 |
| Non-human primate | House dust mite | Plopper and Hyde 2008 |
| | Ascaris | Patterson and Harris 1992 |

1.1.4 Diisocyanate-induced asthma

Patients with occupational asthma comprise 10–15% of all asthma patients (Bardana 2008). Diisocyanates (DI) are used in the synthesis of polyurethane, which is used in paints, adhesives, plastics and foam. The three most used DIs are toluene diisocyanate (TDI), diphenylmethane diisocyanate (MDI), and hexamethylene diisocyanate (HDI). Work-related diisocyanate-induced asthma (DIA) is one of the most common forms of occupational asthma. It has many similarities to atopic asthma, but a poor prognosis despite discontinued exposure to DIs and adequate treatment with ICS (Liu and Wisnewski 2003). DI exposure may result in

hyperreactivity to diisocyanates and NSBHR. The correlation with diisocyanate-specific IgE in serum or total IgE levels is not as strong as in allergic asthma (Liu and Wisnewski 2003, Pronk et al. 2007). Besides biological factors, one reason for the contradictory results might be in the assays used to detect specific IgE (Campo et al. 2007, Ott et al. 2007). The short duration of exposure, IgE positivity to DIs, and low level of NSBHR are factors that seem to predict a good outcome (Padoan et al. 2003, Park and Nahm 1997, Piirila et al. 2000, Pisati et al. 2007, Rachiotis et al. 2007). Elevated IL-8 and neutrophil-derived myeloperoxidase levels are associated with a worse outcome (Maghni et al. 2004). Even after the cessation of exposure and with inhaled corticosteroid treatment, NSBHR, inflammation and symptoms may persist (Padoan et al. 2003, Piirila et al. 2000).

The number and size of biopsy and BAL studies in humans have been limited. Inflammation in the airways has characteristics of atopic asthma, although there is evidence of a mixed Th1/Th2 response (Liu and Wisnewski 2003). An excess of mast cells, eosinophils and activated T cells has been found in the bronchial mucosa (Bentley et al. 1992, Saetta et al. 1992), while an excess of neutrophils and eosinophils has been recorded in BAL (Fabbri et al. 1987). Elevated numbers of CD8+ cells in the bronchial mucosa are a more consistent finding in DIA, while in allergic asthma CD4+ cells are overrepresented (Maestrelli et al. 1994, Wisnewski et al. 2003). Increased expression of TNF- α , IL-1 β , IL-4 and IL-5 has been shown in bronchial biopsies (Jones et al. 2006, Maestrelli et al. 1994, Maestrelli et al. 1995, Maestrelli et al. 1997).

1.1.5 STAT4 and STAT6

Signal transducers and activators of transcription (STAT) are a family of transcription factors important in the signalling of cytokines (Pfitzner et al. 2004). Janus kinases (JAK) are intracellular mediators of receptor binding. Binding of cytokine to its receptor activates JAK, which causes STAT to translocate to the nucleus. STAT4 is a key transcription factor in the development of Th1-type inflammation, while STAT6 is critical for the Th2-type response. Both STAT4 and STAT6 have been shown to play a role in Th2-type inflammation (Akimoto et al. 1998, Hoshino et al. 2004, Raman et al. 2003, Syrbe et al. 2006).

STAT6

STAT6 is activated when IL-4 or IL-13 bind to the α chain of the IL-4 receptor (IL4R α) (Kuperman and Schleimer 2008). Many gene variations of the IL-4 and IL-13 pathways have been linked to asthma. For example, the presence of asthma-associated polymorphisms of IL-13 and IL-4R α together was reported to increase the risk of asthma five-fold in the Dutch population (Howard et al. 2002). STAT6 activates many genes important in Th2-type polarisation and allergic inflammation, including IL-4 (Curiel et al. 1997) and CCL11 (Hoeck and Woisetschlager 2001), and suppresses the expression of IFN- γ . Recently, STAT6-

dependent signalling was shown to induce RhoA expression in cultured human bronchial smooth muscle cells in experimental studies by Chiba et al. (Chiba et al. 2010). RhoA is a small GTP-binding protein that has been suggested to play a role in smooth muscle cell hypercontractility in asthma. STAT6^{-/-} mice have impaired Th2-cell differentiation and IgE-class switching, as well as a decreased response to allergens and parasites (Akimoto et al. 1998, Kaplan et al. 1996, Shimoda et al. 1996, Takeda et al. 1996). STAT6^{-/-} mice exhibit no airway hyperreactivity and eosinophilic inflammation in response to allergen challenge. STAT6 has been found to be increased in the bronchial epithelium in patients with severe asthma (Mullings et al. 2001). Although STAT6 has been under extensive research as a target for new medications for asthma, with promising results (McCusker et al. 2007, Walker et al. 2009), there have been concerns. In a chronic asthma model induced by OVA, STAT6^{-/-} mice unexpectedly had AHR, remodelling comparable to WT mice and Th1 polarisation (Foster et al. 2003). In IL-4/IL13^{-/-} mice, these changes were not seen. Alternative routes may overtake the actions of STAT6 (Zimmermann et al. 2004). An explanation for the controversial results may also be differences between mouse strains in signalling cascades downstream of IL13 and IL4 (Hirota et al. 2009), or different experimental protocols (Olmez et al. 2009). Although IL-4 also has STAT6-independent targets, IL-4 and STAT6 signalling seem to be connected to each other in Th2-type inflammation, while IL-13 has other routes of action (Elo et al. 2010, Foster et al. 2003, Kim et al. 2010b, Sehra et al. 2008, Wills-Karp and Finkelman 2008).

STAT4

STAT4 was first described as a mediator of the effects of IL-12, an important Th1 cytokine (Thierfelder et al. 1996). The most important consequence of STAT4 activation is increased expression of IFN- γ . Accordingly, IFN- γ expression is suppressed in STAT4^{-/-} mice. Thus, the importance of STAT4 in inflammation was first shown in models of autoimmune diseases (Pfitzner et al. 2004), but evidence for its involvement in asthma has now also begun to emerge. Polymorphisms of the STAT4 gene are associated with an increased risk of asthma in the Chinese population (Li et al. 2007, Park et al. 2005a), but not in Finns (Pykalainen et al. 2005). One group has proposed DNA methylation as a way to regulate the expression of both STAT4 and STAT6, instead of allelic variation in T cells (Kim et al. 2010a, Shin et al. 2005). Although the administration of IL-12 or IFN- γ suppresses inflammation and AHR in mouse models of asthma (Behera et al. 2002, Schwarze et al. 1998), the role of STAT4 is more complicated. Cockroach antigen sensitized STAT4^{-/-} mice showed decreased AHR, eosinophilia and chemokine production compared to WT mice, implying a role for STAT4 in allergic inflammation (Raman et al. 2003). Furthermore, IFN γ **has been shown to contribute** to the priming of CD4⁺ cells as IL-4-producing cells (Bocek et al. 2004). Supporting the dual role of STAT4, it seems that inhibition of the IL-12/STAT4/IFN- γ axis during allergen sensitisation leads to an elevated Th2 inflammatory response, but its inhibition during challenge attenuates inflammation (Meyts et al. 2006). A very interesting recent article by

Kim et al. (2010b) suggested that IL-12/STAT4/IFN- γ is a downstream pathway of IL-13-dependent IL-4R α -independent asthma phenotypes such as NSBHR and non-eosinophilic inflammation, suggesting a new role for STAT4 in airway inflammation.

1.2 Remodelling in the airways

1.2.1 General

Although the presence and importance of airway inflammation in asthma is clear, structural changes in the airway wall are also considered important (Holgate 2008). According to the most enthusiastic remodelling researchers, we have come to a dead end in the study of Th2-type inflammation and in trying to identify targets for asthma treatment from signs and consequences of the disease, instead of trying to target the reasons for the observed changes (Holgate and Davies 2009). Remodelling in asthma is the same as the structural changes seen in the airways of asthmatics in comparison to healthy controls. This is the result of coordinated interaction between resident and infiltrating cells and extracellular matrix (ECM) proteins (Holgate 2008). Thus, remodelling is very tightly related to inflammation, although remodelling and inflammation may be seen separately *in vivo*. Remodelling is normally seen during wound healing and development. Classically, remodelling is considered to follow inflammation, as in wound healing (Eming et al. 2007). It has been known for long that the airways do not look the same in fatal asthma compared to those of non-asthmatics, but the actual golden years of remodelling research began a little over a decade ago. Remodelling in the human airways includes changes such as thickening of the airway wall, the deposition of ECM proteins beneath the epithelium and changes in the epithelium (Bousquet et al. 2000, Holgate 2008, Jeffery 2004, Laitinen et al. 2000). An increase and changes in the vasculature and neural networks have also been demonstrated. Thickening of the airway wall is a consequence of many changes, such as an increase in airway smooth muscle (ASM) and ECM proteins, especially the thickening of the bronchial basement membrane (BM), oedema, and an elevated number of mucosal glands. Remodelling is evident in allergic asthma, as well as in DIA (Boulet et al. 1994, Holgate 2008, Saetta et al. 1992, Saetta et al. 1992).

Although not directly associated with the severity of remodelling and all phases of it, the progressive loss of lung function also seen in asthma (Grol et al. 1999, Lange et al. 1998), might be caused by remodelling (Holgate 2008, Pascual and Peters 2005). In HRCT, the airway wall area is inversely correlated with FEV1 and asthma severity in asthma, but not in eosinophilic bronchitis (Niimi et al. 2000, Park et al. 2006). This thickening, causing rigidity into the airway wall, is also regarded as a possible defence mechanism protecting the airways from repeated bronchoconstriction (Holgate 2008).

1.2.2 ASM

ASM hyperplasia and hypertrophy are among the most prominent features in the airway wall and correlate with the severity of disease (Ebina et al. 1993, James and Carroll 2000). Extensive research has been carried out on the mechanisms of ASM modulation, as bronchoconstriction is a cardinal feature of asthma (Dekkers et al. 2009, Halayko and Solway 2001, Panettieri 2003). ASM has two different major phenotypes, contractile and proliferating. ASM from asthmatic individuals has different contractile properties and an increased capacity to proliferate. Although ASM is very plastic, modifying its properties according to external signals (Halayko and Solway 2001), something is different in these cells from patients with asthma compared to those from controls. ASM cells can respond to various stimuli from other cells in asthmatic lung tissues and changes in the ECM. They can secrete a variety of mediators, including cytokines and chemokines, and the ECM. Changes in ASM also seem to be directly associated with inflammation. In addition, inhaled corticosteroids (ICS) probably partly act by affecting muscle, while thermoplasty of ASM, reducing the muscle mass through heating, is under research as a new treatment for asthma (Mauad et al. 2007).

1.2.3 Mediators

In addition to inflammatory mediators, many other mediators of tissue homeostasis have been implicated in asthma pathogenesis, including a disturbed balance between MMPs and their inhibitors (tissue inhibitors of matrix metalloproteinases, TIMP) (Mautino et al. 1999) and disturbed expression of growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and TGF- β (Holgate 2008, Pascual and Peters 2005). TGF- β , a member of the TGF- β superfamily, is among the most studied (Bosse and Rola-Pleszczynski 2007, Howell and McAnulty 2006). Three different splice variants of TGF- β exist, TGF- β 1–3. As with many other growth factors, TGF- β 1 is secreted as a latent form into the ECM, where it attaches. It is activated by several mechanisms, including MMPs, and has numerous activities in cell proliferation and differentiation, immunomodulation, and wound healing. It is a mediator of tissue fibrosis. Recently, TGF- β 1, a master switch of epithelial-mesenchymal transition (EMT), has been proposed to drive EMT of bronchial epithelial cells to mesenchymal cells with myofibroblast characteristics (Doerner and Zuraw 2009). An excess of myofibroblasts has been demonstrated in asthma (Westergren-Thorsson et al. 2010).

1.2.4 Bronchial BM

Pathological changes in asthma include the thickening of the lamina reticularis of the BM, consisting of glycoproteins such as tenascin-C, fibronectin (FN), collagen I, III and V, and proteoglycans (Holgate 2008, Jeffery 2004, Laitinen et al. 2000, Roche et al. 1989).

The thickening is greater in atopic than in non-atopic patients (Amin et al. 2000). It may even be seen after allergen challenge in asthmatics, and persists after the inflammatory response is silenced (Kariyawasam et al. 2007). The thickening is of same magnitude in severe childhood asthma as in adults, and the thickness of the BM is not defined by the duration of the disease (Payne et al. 2003).

The terminology is slightly misleading. When discussing the basement membrane in the lung, it can mean two things: the lamina lucida and lamina densa +/- lamina reticularis. The lamina lucida and lamina densa are the real basement membranes described next, and the lamina reticularis is the ECM, devoid of cells under it. The lamina densa and lamina reticularis are connected through anchoring fibrils of collagen VII. They can be separated in electron microscopy, but not in light microscopy. However, the lamina lucida might be an artefact from the handling of the tissue and lamina densa could be the real structural layer. Basement membranes were characterised between the epithelium and ECM as acellular structures separating these two compartments, but have also been found in other locations, serving the same functions (Kruegel and Miosge 2010, Paulsson 1992, Timpl 1996). They are composed of self-assembling collagen IV and laminin networks connected by other proteins such as nidogen. Although capable of self-assembly, reciprocal interactions with cells are important for the formation of BMs. Collagen IV is essential for the stability of BMs, while laminins are essential for their assembly. Other major components include proteoglycans high in heparin sulphate, although numerous molecules have been recognised. Cells bind and recognize constituents of the BMs through cell surface receptors such as integrins. The composition of basement membranes is tissue- and time-specific. They are not just barriers, scaffolds for attachment and filters, but are involved in many processes such as cell migration, development, growth, signalling and differentiation. Changes in basement membrane composition have been implicated in various diseases, including cancers.

There is a layer of flat fibroblasts directly under the BM in asthmatic as well as in normal subjects, which may serve as one means of communication between the epithelium and lamina propria, may transform to myofibroblasts in response to injury to the epithelium (Morishima et al. 2001), and may be responsible for the thickening of the lamina reticularis (Evans et al. 1999).

1.2.5 Epithelium

Shedding of the epithelial cells is a common finding in asthma, but not seen in patients with the mildest form of the disease (Jeffery 2004, Laitinen et al. 1985). One **current view** introduced (or reintroduced) at the beginning of the 21st century proposes that the initial damage is in the epithelium, which guides the mesenchyme (together called the epithelial-mesenchymal trophic unit, EMTU), and the view has since been refined (Holgate et al.

2000, Holgate et al. 2004, Holgate and Davies 2009, Holgate et al. 2009). The damaged epithelium begins to produce a pool of cytokines, chemokines and growth factors. The barrier function is also jeopardized. The initial damage might result from factors such as virus infection or pollutants in wrongly programmed epithelium. These, in turn, activate the ECM/mesenchymal cells, including dendritic cells, fibroblasts and smooth muscle, and attract and activate inflammatory cells. The result is chronic inflammation comprising a constant excess of growth factor receptors, increased cytokine and growth factor production, infiltration of inflammatory cells and Th2 polarization. The view is completed by the idea of improper activity of genes that were supposed to be silenced after birth, since many of the mediators involved in asthma are also important during morphogenesis (Bousquet et al. 2004). This leads in time to a dynamic state named remodelling, including pathological changes in epithelial cells and the ECM as this one type of chronic wound healing continues (Bousquet et al. 2004, Laitinen et al. 2000). Thymic stromal lymphopoietin (TLSP), which is secreted by bronchial epithelial cells in response to threats and damage *in vitro* (Allakhverdi et al. 2007), is able to induce a Th2-type response, promoting DCs and mast cells (Allakhverdi et al. 2007, Soumelis et al. 2002). Furthermore, asthmatics have been reported to display increased expression of TLSP in biopsies (Ying et al. 2005).

Localization of the expression of many asthma susceptibility genes to the mesenchyme and epithelium has supported this view of the EMTU (Holgate et al. 2009). One gene with probably the strongest evidence and most positive studies is a member of the membrane bound metalloproteases, a disintegrin and metalloproteinase domain 33, *ADAM33* (Holgate et al. 2006, Van Eerdewegh et al. 2002). It is preferably expressed in mesenchymal cells in adults, especially in smooth muscle, and during morphogenesis. Its expression is increased in asthma and has been implicated in pathogenesis (Haïtchi et al. 2009, Jie et al. 2009). To further strengthen this view, inflammation and remodelling already exists in infants who will develop asthma in the future (Baena-Cagnani et al. 2007). Furthermore, glucocorticoid treatment of children with atopic parents or only symptoms of asthma but no asthma diagnosis do not reverse the natural progression of the disease (Holgate 2008). Some other theories concentrated more on inflammation have also considered the epithelium as the origin (Holtzman et al. 2002, Lambrecht and Hammad 2010).

1.2.6 Mouse models

Remodelling in the lung has been shown in a chronic allergic mouse model, but structural changes in the acute phase have, as expected, been minimal (Christie et al. 2004, Kumar et al. 2004, McMillan and Lloyd 2004). Remodelling persists in the mouse lung, even after antigen exposure is terminated and inflammation disappears (Kumar et al. 2004). In addition to the larger airways, remodelling changes in mouse lung have also appeared in the lung parenchyma (Hirota et al. 2006, Xisto et al. 2005). Thus, mice might make a good model

for studying the mechanisms of remodelling. However, remodelling has always been a consequence of artificial sensitisation and inflammation as in classical view of remodelling, because mice do not naturally develop asthma.

1.2.7 Tenascin-C

Tenascins are ECM glycoproteins. Four isoforms have been recognised in vertebrates: tenascin-C, tenascin-X, tenascin-R and tenascin-W (Brellier et al. 2009, Jones and Jones 2000, Tucker and Chiquet-Ehrismann 2009). Tenascin-Y is the avian homologue of tenascin-X. Each of the tenascins has a unique temporal and spatial expression pattern. They consist of similar motifs: 1) heptad repeats (an amino-terminal oligomerization domain), 2) EGF-like repeats, 3) FN type III repeats, and 4) a fibrinogen-like globular carboxy terminal. Tenascin-C polypeptides form a hexabrachion, which was first discovered from the tenascin family. Tenascin-C has different splice variants, resulting in different compositions of FN III repeats in a species-, tissue- and time-specific manner. Because it is usually expressed quite transiently, the expression pattern has been relatively difficult to study. Its expression can be modulated by many factors, including growth factors, mechanical stress, inflammatory mediators and MMPs, which are all involved in the pathogenesis of asthma. Depending on the molecules tenascin-C interacts with, it can promote adhesion, cell migration or growth, or inhibit all of the above (Jones and Jones 2000). Tenascin-C is usually quite antiadhesive and promotes the detachment and migration of cells. Thus, in addition to morphogenesis, it is very important in cancer development, wound healing and inflammatory diseases.

Tenascin-C participates in development and remodelling in the lung (Jones and Jones 2000, Roth-Kleiner et al. 2004). In the developing lung, tenascin-C is needed for branching morphogenesis of the bronchial tree (Kaarteenaho-Wiik et al. 2001, Lambropoulou et al. 2009, Roth-Kleiner et al. 2004, Young et al. 1994). It is first expressed more in mesenchymal cells, then in the canalicular phase in and near epithelial cells, after which expression is gradually down-regulated. It almost disappears after alveolarisation (Kaarteenaho-Wiik et al. 2001, Lambropoulou et al. 2009). In human adult bronchi, BM is negative or faintly positive for tenascin-C in healthy controls, but its expression is markedly increased in asthma in the stable phase and even immediately after specific antigen challenge (Amin et al. 2000, Kariyawasam et al. 2007, Laitinen et al. 1997, Phipps et al. 2004, Weinacker et al. 1995). The accumulation of tenascin-C is stronger in atopic than in nonatopic asthma patients and correlates with inflammatory cell numbers in the lamina propria (Amin et al. 2003, Karjalainen et al. 2003). The tenascin-C-positive percentage of the lamina reticularis has been shown to correlate with the recovery from histamine challenge (Tsurikisawa et al. 2010). A coding single nucleotide polymorphism (SNP) in an alternatively spliced FNIII repeat of tenascin-C has been linked to adult asthma in the Japanese population (Matsuda et al. 2005). Furthermore, neuropeptide S – neuropeptide S receptor 1 (NPSR1) signalling increases tenascin-C expression, and the NPSR1 gene is a susceptibility gene for asthma (Orsmark-Pietras et al. 2008).

In an OVA-induced mouse model of allergic airway inflammation and in patients with atopic asthma, the Th2 type of inflammation prevails in the airways. It has been shown that leukotrienes, IL-13, IL-4, TNF- α and IFN- γ upregulate tenascin-C production *in vitro* (Altraja et al. 2008, Harkonen et al. 1995, Lee et al. 2001b, Rettig et al. 1994, Syed et al. 2005, Yuyama et al. 2002). Furthermore, tenascin-C can modulate cytokine expression (Midwood and Orend 2009). Glucocorticoid treatment has been shown to reduce the thickness of the tenascin-C layer (Laitinen et al. 1997). OVA induces tenascin-C expression and Th2-type inflammation, while tenascin-C-deficient mice show attenuated Th2- type inflammation in the lung in response to OVA (Nakahara et al. 2006). Thus, it appears that tenascin-C might have multiple roles in inflammation in the airways. It might be more an inflammatory mediator than a structural protein in adults. TGF- β is also a known inducer of expression for tenascin-C (Jones and Jones 2000). TGF- β is **known to bridge the transition from Th2-type inflammation** to fibrosis, but independent mediators exists (Lee et al. 2001a, Takayama et al. 2006). The cyclic strain that is exerted on the airway wall during bronchoconstriction induces tenascin-C production through changes in the actin cell skeleton (Sarasa-Renedo et al. 2006).

1.2.8 Laminins

Laminins are large heterotrimeric glycoproteins composed of one α , one β and one γ chain, forming cruciform, T-shaped or rod-like structures. Five α chains, three β chains and three γ chains have been identified in vertebrates that actually participate in the formation of known laminins (Aumailley et al. 2005, Colognato and Yurchenco 2000, Durbeej 2010, Miner 2008, Scheele et al. 2007). At least 15 laminin trimers have been discovered. The nomenclature is presently systematic, as can be seen in Table 4 (Aumailley et al. 2005). For instance, laminin composed of $\alpha 1$, $\beta 1$ and $\gamma 1$ chains is named laminin 111. A and B are used to designate splice variants of the chains. Laminins are situated in the BM in various tissues. They self-polymerise under the epithelium, which is guided by α chains and connections to cells. Similarly to tenascins, laminins have a tightly regulated temporospatial expression pattern. They are important for tissue homeostasis, since they participate in directing cell differentiation, movement, shape and survival. They may even promote bacterial invasion. Laminins communicate with cells through integrin and non-integrin receptors on the cell surfaces. Laminins and their interactions with cells have gained considerable interest among cancer researchers. Mutations in laminin genes may lead to serious defaults in phenotypes. A deficiency of laminin $\alpha 2$ may cause muscular dystrophy (Guo et al. 2003), and a deficiency of laminin $\beta 2$ congenital nephrotic syndrome with neuromuscular and ocular defects (Zenker et al. 2004). Mutations in human LAMA2 and LAMB2 genes associated with the diseases have been found for both (Matejas et al. 2010, Oliveira et al. 2008).

In healthy adult human lungs, $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 3$, $\gamma 1$, $\gamma 2$ chains have been revealed in the bronchial BM (Amin et al. 2005, Petajaniemi et al. 2002, Virtanen et al. 1996, Virtanen et al. 2000).

Because of problems with antibody specificity, some older papers claim expression of laminin $\alpha 1$ chain in adults in the bronchial BM, but this is actually laminin $\alpha 5$. The laminin $\alpha 5$ chain and laminin 111, at least, are critical for lung development (Nguyen and Senior 2006). During morphogenesis, $\alpha 2$ and $\beta 2$ chains are also expressed under the bronchial epithelium. The laminin $\alpha 2$ chain disappears during the early pseudoglandular stage of development, while laminin $\beta 2$ is expressed at least until the end of the canalicular period. During the former, branching morphogenesis and cell type specialisation occur, while in the latter phase the real respiratory portion of the lungs begins to form.

Table 4. Nomenclature for laminins

| <i>Laminin trimers</i> | <i>Abbreviated</i> | <i>Previous abbreviation</i> |
|----------------------------|----------------------|------------------------------|
| $\alpha 1\beta 1\gamma 1$ | Laminin-111 | Laminin-1 |
| $\alpha 2\beta 1\gamma 1$ | Laminin-211 | Laminin-2 |
| $\alpha 1\beta 2\gamma 1$ | Laminin-121 | Laminin-3 |
| $\alpha 2\beta 2\gamma 1$ | Laminin-221 | Laminin-4 |
| $\alpha 3A\beta 3\gamma 2$ | Laminin-332 or -3A32 | Laminin-5 or 5A |
| $\alpha 3B\beta 3\gamma 2$ | Laminin-3B32 | Laminin-5B |
| $\alpha 3A\beta 1\gamma 1$ | Laminin-311 or -3A11 | Laminin-6 or -6A |
| $\alpha 3A\beta 2\gamma 1$ | Laminin-321 or -3A21 | Laminin-7 or -7A |
| $\alpha 4\beta 1\gamma 1$ | Laminin-411 | Laminin-8 |
| $\alpha 4\beta 2\gamma 1$ | Laminin-421 | Laminin-9 |
| $\alpha 5\beta 1\gamma 1$ | Laminin-511 | Laminin-10 |
| $\alpha 5\beta 2\gamma 1$ | Laminin-521 | Laminin-11 |
| $\alpha 2\beta 1\gamma 3$ | Laminin-213 | Laminin-12 |
| $\alpha 4\beta 2\gamma 3$ | Laminin-423 | Laminin-14 |
| $\alpha 5\beta 2\gamma 3$ | Laminin-523 | Laminin-15 |

Adapted from Aumailley et al. (2005)

Laminin $\alpha 2$ and $\beta 2$ chains re-emerge in asthma (Altraja et al. 1996). The laminin $\beta 2$ chain is sometimes seen in healthy controls, but laminin $\alpha 2$ is only evident in asthma. Laminin $\alpha 2$ is usually expressed together with laminin $\beta 2$, while laminin $\beta 2$ is often expressed alone. Laminin $\alpha 2$ is a selective adhesion substrate for myofibroblasts in foetal mouse lung, as fibronectin serves as a platform for the whole mesenchymal cell population *in vitro* (Flores-Delgado et al. 1998). The stretch-induced expression of the laminin $\alpha 2$ chain is known to promote the transition of mesenchymal cells to the smooth muscle cell phenotype by down-regulation of RhoA, which is a GTPase needed in the reorganisation of the actin cytoskeleton (Beqaj et al. 2002, Relan et al. 1999). Binding to endogenous laminin seems to be needed for changes in mature ASM cells in (Tran et al. 2006). No actual association between increased numbers of myofibroblasts and the laminin $\alpha 2$ chain has been shown in asthma. No functional results on the importance of laminin $\beta 2$ in the development of the lungs have, to my knowledge, been published. The laminin $\alpha 2$ chain is usually not expressed alone in asthma, but always with the $\beta 2$ chain (Altraja et al. 1996). In congenital muscular

hypertrophy, a deficiency of the laminin $\alpha 2$ chain reduces the expression of laminin $\beta 2$, probably reflecting the guidance of laminin assembly by α chains (Cohn et al. 1997).

1.2.9 Relationship between inflammation and remodelling

In mouse models, inflammation precedes remodelling and requires long-term challenge (Kumar et al. 2004). As described above, the concepts of asthma pathogenesis based mainly on the mechanisms found in mouse models also consider inflammation to precede remodelling in humans (Holgate 2008). However, the order of appearance of inflammation and remodelling in human asthma has not been definitively established (Baena-Cagnani et al. 2007). For instance, children with respiratory symptoms destined to develop asthma already have remodelling changes in the lung (Pohunek et al. 2005). The role and the exact timing of the remodelling in the pathogenesis of asthma remain to be elucidated.

2. Aims of the study

The overall aim of the research presented in this thesis was to investigate airway inflammation and the expression of laminins and tenascin-C in airway remodelling in different types of asthma, and in asymptomatic smokers in comparison to healthy non-smokers. We hypothesized that:

- I) asymptomatic smokers would have disturbed inflammatory balance in the airways compared to non-smokers;
- II) airway inflammation in diisocyanate-induced asthma (DIA) patients with persistent NSBHR would differ from that in patients without NSBHR after the cessation of exposure and treatment with ICS;
- III) we would find *in vivo* regulators of tenascin-C expression in asthma by investigating the association between Th polarisation and tenascin-C expression in an animal model of allergic asthma utilizing STAT4 and STAT6 knockout (KO) mice; and
- IV) we could relate the development of clinical asthma to the laminin expression profile in the bronchial BM by studying subjects with newly diagnosed asthma, those with asthma symptoms but not fulfilling the criteria for an asthma diagnosis and healthy controls.

3. Materials and methods

The animal studies were approved by Ethics Committee of the Finnish Institute of Occupational Health (FIOH) (Helsinki, Finland) and the State Provincial Office of Southern Finland (Helsinki, Finland). The human studies were approved by the Ethics Committee of the Department of Medicine, Helsinki University Central Hospital (Helsinki, Finland), the Ethics Committee of the North Karelia Central Hospital (Joensuu, Finland) and the Ethics Committee on Human Research at the University of Tartu (Tartu, Estonia). All subjects gave informed consent

3.1 Subjects (I–II, IV)

3.1.1 Healthy subjects, asymptomatic ex-smokers and smokers

The healthy controls were volunteers from the regions of Helsinki University Central Hospital (HUCH) (n = 15) (I–II) and Tartu University Hospital (n = 8) (IV). Common exclusion criteria were a pulmonary or other disease, atopic symptoms, and an acute respiratory infection within the previous 4 weeks. All the healthy subjects included had spirometry results within normal limits, normal laboratory tests and no allergies. Allergies and atopy were excluded by history and negative skin prick tests (IV) or IgE measurements (I–II). In addition, subjects underwent a histamine provocation test to exclude BHR (I–II) or performed a 2-week PEF follow-up (IV). Among the controls recruited from the HUCH region (I–II), there were 5 smokers, 2 ex-smokers and 8 non-smokers. The controls from Tartu (IV) were all non-smokers.

3.1.2 Patients

We studied altogether 67 asthmatics of various types, who were divided into the following three groups:

Patients with DIA (n = 17) (II) were recruited at FIOH. The diagnosis was made after specific challenge with the DI to which patient had been exposed to, as described earlier (Piiirila et al. 2000). Exposure to DIs was terminated after the diagnosis or before because of respiratory symptoms. This was a follow-up study, and patients went on budesonide 1600 µg/day (Astra Zeneca, Södertälje, Sweden) after the first bronchoscopy. Some patients had been using ICS before the study and the medication was discontinued for 17.5 d (mean, range 6–60) before the first examination. After 1 year, adjustments of the medication were allowed according to the patients' needs. Examinations were repeated on study visits after 6 months and 2–3

years. Smoking was permitted: 4/17 patients were smokers and 7/17 were ex-smokers. None of the patients changed their smoking habits during the study.

Patients with symptoms but without an asthma diagnosis ($n = 15$) (IV) were recruited from the region of HUCH. Inclusion criteria were an elevated serum ECP concentration ($> 500 \mu\text{g/l}$), asthma symptoms (wheezing, cough or dyspnoea), but no asthma according to lung function tests. Asthma was excluded by PEF follow-up with diurnal variation $<20\%$ and FEV1 and PEF reversibility $<15\%$. Exclusion criteria were significant systemic disease and respiratory infection within the 4 weeks before the study visit. The patients with asthma symptoms used no inhaled or oral steroids, cromones, methylxanthines or long-acting β_2 -agonists for 4 weeks, no long-acting antihistamines or nasal corticosteroids for 6 weeks and no short-acting antihistamines for 2 weeks before the study.

Patients with mild asthma ($n = 35$) (IV) had received an asthma diagnosis <2 years previously. The diagnosis was made according to international criteria (National Heart Lung and Blood Institute, National Asthma Education and Prevention Program. April 1997). Patients were recruited from the regions of HUCH and the North Karelia Central Hospital. Additional inclusion criteria were active asthma symptoms and BHR 1 week before or at the study visit. Patients with asthma exacerbation during the preceding four weeks were excluded. The asthma medications were discontinued as follows: long-acting β_2 -agonists or leukotriene inhibitors for 4 weeks, antihistamines for 2 weeks and inhaled or oral steroids for the 2 months before the study. Atopy was screened by skin prick testing.

3.1.3 Lung functions

All participants performed a spirometry according to international standards (Quanjer et al. 1993). The diffusing capacity and total lung capacity were measured from all participants in studies I and II. Finnish reference values of Viljanen (Viljanen et al. 1982) were used in both aforementioned procedures. NSBHR was assessed by a histamine challenge test according to Sovijärvi et al. (Sovijarvi et al. 1993). In addition, DIA patients (II) were challenged with DIs and control substances to confirm the diagnosis 48h before the bronchoscopy as described earlier (Piirila et al. 2000).

3.1.4 Bronchoscopy

Fibreoptic bronchoscopy (Olympus BF-XT30) was performed according to international guidelines (National Institutes of Health 1991) after local anaesthesia with lidocaine. BAL was sampled according to Taskinen et al. (1992) and the bronchial biopsies were taken with cupped forceps from untouched areas.

3.2 OVA- induced mouse model of allergic airway inflammation (III)

3.2.1 Animals

Wild-type (WT) Balb/c mice were purchased from M&B (Denmark). STAT4^{-/-} and STAT6^{-/-} mice of the same Balb/c background were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). All the mice were housed at the Finnish Institute of Occupational Health under pathogen-free conditions and kept on an OVA-free diet.

3.2.2 Sensitisation and challenge

All the mice were sensitised with intraperitoneal (i.p.) injection of 50 µg of OVA emulsified in aluminium hydroxide on days 0 and 14. On days 28, 29 and 30 the mice were challenged with nasally inhaled phosphate buffered saline (PBS) (controls) or 50 µg of OVA under isoflurane inhalation anaesthesia.

3.2.3 Airway responsiveness and sample collection and preparation

A single-chamber whole-body plethysmograph (WBP) (Buxco Technologies, Troy, NY, USA) was used to assess airway responsiveness 24 hours after the last airway challenge as described earlier (Hamelmann et al. 1997). Unrestrained mice were first exposed to nebulised PBS to record the baseline enhanced pause (Penh) values, and then to increasing concentrations of metacholine (Mch) (Sigma-Aldrich, UK). Airway responsiveness was expressed as absolute Penh values. Penh is a value obtained through a mathematical formula based on the flow curve of WBP.

After airway challenge, the mice were killed by isoflurane overdose. The hepatic vein was drained, the chest cavity opened and the lungs were lavaged. A fraction of the bronchoalveolar lavage (BAL) sample was cytopspun on a slide and stained with MayGrünwald-Giemsa (MGG) stain for inflammatory cell counting. Half of the right lung embedded in TissueTek OCT Compound and the left lung were quick-frozen for immunohistological and RNA studies, respectively. The other half of the right lung was fixed in paraformaldehyde and embedded in paraffin.

3.3 Immunohistochemistry

The primary antibodies used in this research with working dilutions are listed on Table 5. All the immunohistochemical studies were carried out with quick-frozen specimens, except the measurement of the tenascin-C layer in DIA patients (unpublished). The tissue integrity was better in paraffin-embedded biopsies, but there were much greater problems with antibody binding. Quick-frozen samples were sectioned at 6 µm with a cryostat and paraffin blocks at room temperature (RT) onto glass slides. The integrity of the lung samples was confirmed with routine haematoxylin-eosin staining. Negative controls were handled as follows, but the primary antibodies were omitted. All the antibodies have been characterised and most of them have been used for a number of years in laboratory research at the Department of Anatomy of the Institute of Biomedicine, University of Helsinki.

3.3.1 Tenascin-C and laminins (III–IV and unpublished data)

Indirect immunofluorescence was used to visualize antibody binding to tenascin-C and laminins. For indirect immunofluorescence, slides were fixed with acetone at -20 °C for 10 min and rinsed in PBS. The slides were then incubated with primary antibody at RT for 30 min followed by rinsing and incubation with the appropriate FITC-conjugated IgG secondary antibody (Jackson ImmunoResearch Laboratories Europe, UK). Finally, the slides were rinsed and mounted in sodium veronal buffered glycerol. Tenascin-C immunoreactivity was graded in the mouse lung from 0–4 as follows: 0 = no reactivity, 1 = weak reactivity, 2 = intermediate reactivity, 3 = strong reactivity, 4 = very strong reactivity. All the samples were coded.

Paraffin-embedded sections were used to measure the thickness of the tenascin-C layer in DIA patients and controls. First, the slides were deparaffinised in xylene and hydrated in a degrading alcohol series. Pepsin treatment was used for antigen retrieval. The APAAP protocol (below) was then followed without the fixing. The sections were photographed under a light microscope and the open source software ImageJ was used to measure the thickness of the tenascin-C layer.

Table 5. Monoclonal antibodies used in this research

| <i>Antigen</i> | <i>Antibody</i> | <i>Ref./Company</i> | <i>Dilution</i> | <i>Studies</i> |
|--|-------------------|--|-----------------|----------------|
| Cleaved ECP (eosinophils) | EG2 | Pharmacia, Uppsala, Sweden | 1:50 | I,II |
| Mast cell tryptase (mastcells) | AA1 | Dako | 1:500 | I,II |
| CD 163 (macrophages) | Ber-MAC3 | Dako | 1:50 | I,II |
| Neutrophil elastase (neutrophils) | NP57 | Dako | 1:2000 | I,II |
| CD3 (lymphocytes) | T3-4B5 | Dako | 1:1000 | I,II |
| CD4 | MT310 | Dako | 1:40 | I,II |
| CD8 | DK25 | Dako | 1:1000 | I,II |
| Recombinant human IL-4 | 1842-01 | Genzyme, Cambridge, MA, USA | 1:50 | I,II |
| Tenascin-C | 100EB2 | Howeedy et al. 1990 | none | III |
| Tenascin-C paraffin embedded samples | 143DB7 | Soini et al. 1992 | 1:1 | up |
| Tenascin-C (mouse) | MTn-12 | Sigma-Aldrich, Aufderheide and Ekblom 1988 | 1:200 | III |
| Laminin α 2 chain | 5H2 | Leivo and Engvall 1988 | 1:800 | IV |
| Laminin α 2 chain (mouse) | 4H82 | Sorokin et al. 1997 | 1:2 | up |
| Laminin α 3 chain | BM2 | Rousselle et al. 1991 | 1:400 | IV |
| Laminin α 5 chain | 4C7 | Engvall et al. 1986, specificity Tiger et al. 1997 | 1:1000 | IV |
| Laminin β 1 chain | 114DG10 | Virtanen et al. 1997 | none | IV |
| Laminin β 2 chain | C4 | Hunter et al. 1989 | 1:2000 | IV |
| Laminin β 2 chain (mouse) | lam β 21117 | Sasaki et al. 2002 | 1:1600 | up |
| Laminin β 3 chain | 6F12 | Marinkovich et al. 1992 | 1:300 | IV |
| Laminin γ 1 chain | 2E8 | Engvall et al. 1990 | 1:300 | IV |
| Laminin γ 2 chain (inlaminin-332) | GB3 | Matsui et al. 1995 | 1:4 | IV |

In parentheses is given either the cell type that the antibody was used to detect or the target species if other than human. up = unpublished data

3.3.2 Inflammatory cells in biopsies (I–II)

The alkaline phosphatase–anti-alkaline phosphatase (APAAP) (Dako, Glostrup, Denmark) technique was used to detect inflammatory cells in biopsies, except eosinophils. For APAAP, sections were fixed in acetone at RT for 10 min and rinsed with Tris-buffered saline between every step in the procedure. They were incubated with the primary antibody for 1 h followed by the APAAP protocol according to the manufacturer's instructions. New Fuchsin (Sigma, St Louis, MO, USA) was used for colour development. Levamisole was used to block endogenous alkaline phosphatase activity. Slides were counterstained with haematoxylin and mounted in Eukitt mounting medium (Fluka Biochemika, Buchs, Switzerland). To visualize antibody binding to eosinophil cationic protein, the Vectastain Elite avidin-biotin complex (ABC) kit (Vector Laboratories, Burlingame, CA, USA) with DAB development was used according to manufacturer's instructions. Sections for ABC were fixed with paraformaldehyde. Slides were counterstained with haematoxylin and mounted in Eukitt (Fluka BioChemika, Buchs, Switzerland).

Cell densities were counted with the Autocad program 10.1 (Autodesk Inc., Sausalito, CA, USA), as described earlier (Laitinen et al. 1997).

3.4 Real-time quantitative RT-PCR (I–III)

Total RNA from the cells and the lungs was extracted using Trizol Reagent (Gibco BRL, Grand Island, NY, USA) (I–II) or Eurozol Reagent (Euroclone, Italy) (III) according to the manufacturer's protocol. In studies I–II the total RNA was handled with DNAase I (Roche, Munich, Germany) to remove the contaminating DNA. The total amount of RNA was measured with spectrophotometry. RNA was reverse transcribed into cDNA with MultiScribe reverse transcriptase or a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), with random hexamers as primers, according to manufacturer's instructions.

The relative quantitative real-time polymerase reverse transcriptase chain reaction (RT-PCR) was performed with an Applied Biosystems AbiPrism 7700 Sequence Detector System or 7500 Fast Real-Time PCR system according to the manufacturer's instructions. TaqMan probes were used. At the 5' end of the Taqman probe is attached a reporter dye, a fluorophore (in our probes FAM or VIC), and at the 3' end a quencher (in our probes TAMRA). As long as the quencher and fluorophore are close to each other, fluorescence signals by the fluorophore are quenched by the quencher. As the polymerisation proceeds, the probe attached to the template is cleaved and fluorophore is free to emit fluorescence. Thus, following up the accumulation of PCR products is possible in real time by continuous monitoring of the increase in fluorescence of the reporter dye (FAM). More PCR products mean more cDNA template. VIC-labelled ribosomal RNA 18S was used as an endogenous control for each sample to ensure that the samples were comparable with each other. The fluorescence signals were also standardized to the internal passive reference ROX to correct non-PCR-related fluorescence fluctuations. The comparative threshold cycle (Ct) method was used for relative quantification. The threshold was manually adjusted based on amplification curves. The difference between the threshold cycles of the target reporter dye and 18S for each sample was calculated (dCts). The threshold cycle is the number of the PCR cycle when the reporter dye fluorescence is greater than the adjusted threshold, i.e. the accumulation of PCR products is significant. The corresponding calibrator value (dCtc) was subtracted from dCts, resulting in ddCt, which was used in formula 2^{-ddCt} . Results were represented either as these values (RU = relative expression unit) or as fold differences compared to controls.

All the primers and probes were purchased from Applied Biosystems. Human predeveloped PCR primers and probes were obtained as separate assay reagents (eotaxin, RANTES, IL-18, IL-13, IL-6, TNF beta, MIP 3 α , MDC, MIP 1 beta, IL 16, MIP 1alpha, TGF- β 1 and 18 S rRNA) or were preattached to the Cytokine Gene Expression Plate I (IL-1 α , IL-1beta, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN- γ , and TNF- α). Murine PCR primers and probes for cytokines (TNF- α , IL-4 and IFN- γ) were also predeveloped. Laminin and tenascin-C primers and probes were generated with PrimerExpress version 1.5 software and ordered from Applied Biosystems. The sequences for self-designed laminin and tenascin-C primers and probes are listed in Table 6.

Table 6. Self-designed mouse primers and probes

| <i>Target</i> | <i>5'-Forward primer-3'</i> |
|----------------------------------|---|
| | <i>5'-Reverse primer-3'</i> |
| | <i>5'-Probe-3'</i> |
| Tenascin-C (Tnc) | ACC ATG CTG AGA TAG ATG TTC CAA A CTT GAC AGC AGA AAC ACC AAT CC ACC ACA CTC ACA GGT CTA AGG CCC GG |
| Laminin α 2 chain (Lama2) | CTG CTG TCT TAT CTT GAT TTC CTAAGG GAG GAC CAA CTC CAG AGC AGAAT TAT GGC TTC AGC ATC CTG GAC AAG CC |
| Laminin β 2 chain (Lamb2) | CGT GAG GAC TGC ATT CAG AAC TC GCG TGT CCG CCT TGA TCT A CAC TGA CAT GGC AAG GCG CCA TT |

3.5 Cells (III)

MRC-9 cells, purchased from ATCC (Manassas, VA, USA), were cultured in MEM supplemented with 10% FBS, 2 mM glutamine, 0.1 mM nonessential amino acids, 1.5 g/L of sodium pyruvate and antibiotics (Gibco, Invitrogen, Life Technologies, Carlsbad, CA, USA) at 37 °C in 5% CO₂. Cells were stimulated with TNF- α (10 ng/ml) (Biosource International, Camarillo, CA, USA) at 37 °C for 3 and 9 hours.

Primary human dermal fibroblasts isolated from adult skin were purchased from Gibco and cultured in Medium 106 (Gibco) supplemented with LSGS (low serum growth supplement) and antibiotics (Gibco) at 37 °C in 5% CO₂. Cells were stimulated with TNF- α (20 ng/ml), IFN- γ (500 IU/ml, Immuno Tools, Friesoythe, Germany) and with both for 2, 6 and 18 hours.

3.6 Western blotting (III)

Western blotting from whole cell extracts and concentrated cell supernatants was performed to show differences in tenascin-C expression in cell culture. Human primary fibroblasts stimulated with TNF- α and IFN- γ were lysed in protein lysis buffer containing 10 mM Tris

(pH 7.4), 150 mM NaCl and 25% ethylene glycol supplemented with complete mini protease inhibitor mixture (Roche Diagnostic, Indianapolis, IN). The cell extract was homogenized with ultrasound sonicator (Sanyo Electronics, San Diego, CA). Proteins in cell lysates and cell culture supernatants were separated with SDS-PAGE on 12% gels and transferred onto Immobilon-P Transfer Membranes (Millipore). Membranes were blocked with 5% non-fat milk in PBS. Membranes were incubated with monoclonal mouse antihuman antibody against tenascin-C followed with HRP-conjugated polyclonal goat anti-mouse Igs (Dako). Proteins were visualized by a Luminescent Image Analyzer (Image Quant LAS4000mini, GE Healthcare, Sweden).

3.7 Statistics

The data were analysed with GraphPad Prism software (GraphPad Software, Inc., CA, USA) and/or SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Comparisons between groups were performed with the Kruskal-Wallis test, the Mann-Whitney U-test or the unpaired two-tailed Student's t-test when appropriate. To assess the correlation between parameters, Spearman correlation coefficients were determined (I). The Wilcoxon one-sample signed rank test was used when comparing patients' results between examinations (II). When several simultaneous comparisons were made, Bonferroni's correction of the p-values was applied (II). The chi-squared test was applied to compare the expression of the Lm chains between the two patient groups (IV). Values for measurements are expressed as the mean \pm the standard error of the mean (SEM) (III) or the standard deviation (SD) (I–II) and the median with the range (II). P values < 0.05 were considered significant. Bonferroni-corrected levels of significance are reported when necessary.

4. Results and discussion

4.1 Inflammation

4.1.1 Asymptomatic smokers (I)

The effect of chronic smoking on inflammation of the airways before the development of COPD has not been very extensively studied and the results have been contradictory. Thus, we compared the numbers of inflammatory cells and mRNA expression of inflammatory mediators in the BAL samples between non-smokers and asymptomatic smokers.

BAL cells – increased number of macrophages

The number of macrophages was significantly higher in the BAL samples of smokers compared to non-smokers, which is consistent with earlier studies (Barbers et al. 1987, Hagiwara et al. 2001, Kotani et al. 2000, Linden et al. 1993). *In vitro*, mouse alveolar macrophages and human peripheral blood monocytes were shown to be able to adhere to collagen IV, an essential component of the bronchial membrane, which had been altered by smoke extract compounds (Kirkham et al. 2003). This presents a subtle way for macrophages to gather in the airways of smokers. Macrophages are central to the development of COPD, as they participate in innate and adaptive immunity and secrete MMPs, directly destroying the tissue (Tetley 2002). We found no difference between the groups in the numbers of lymphocytes, neutrophils or eosinophils in the BAL. The CD4+/CD8+ ratio was also similar. In previous studies, the ratio of CD4+ to CD8+ cells has been shown to decrease in asymptomatic smokers (Ekberg-Jansson et al. 2000, Wallace et al. 1994). An elevated number of CD8+ cells is a key feature of COPD (Cosio and Agusti 2010).

Attenuated expression of mRNA for inflammatory mediators

mRNA expression for 8 out of 23 studied inflammatory mediators in BAL cells was lower in asymptomatic smokers than in non-smokers (Table 7). Cytokines with very low mRNA expression near the detection limit were not included when interpreting the results. As the macrophages were the largest cell population and we evaluated the relative quantity of mRNA in BAL cells, not the protein in BALF, it can be assumed that most of the attenuated expression of inflammatory mediators was due to macrophages with impaired function. Protein might additionally be secreted to the airway lumen by stationary cells. We found also a negative correlation of cytokines and chemokines with decreased expression in smokers and the number of macrophages in BALF. A positive correlation was detected between FEV1 as a percentage of the predicted value and the cytokines and chemokines. In the following, those cytokines and chemokines with a lower level of expression in smokers than in non-smokers as well as those most relevant to the pathogenesis of COPD are discussed.

Among the studied CC chemokines, mRNA expression of CCL3, CCL4, CCL5 and CCL20 was suppressed in smokers compared to non-smokers. However, results from previous studies contradict our findings. The BALF CCL4 protein content has been shown to be similar in smokers and non-smokers, while CCL3 has been undetectable (Capelli et al. 1999). We examined changes in mRNA expression in BAL cells, which might explain the differing results. In another study, mRNA expression of CCL3 was increased in the bronchial epithelium but not in macrophages in smokers with airflow limitation in comparison to those without airflow limitation and non-smokers (Fuke et al. 2004). In our study, the majority of the cells were macrophages. Furthermore, in the study of Fuke et al. (2004), many of the patients were ex-smokers and were operated because of other lung pathology. In our study, ex-smokers were excluded and none of the subjects had any pulmonary symptoms or disease. The expression of CCL5 mRNA and the number of eosinophils has been found to be strongly increased in chronic bronchitis during exacerbations and slightly increased in patients with stable chronic bronchitis in comparison with healthy non-smoking controls in bronchial biopsies (Zhu et al. 2001). CCL4 and CCL5 are ligands for CCR5, and CCL20 binds to CCR6. CCR5- and CCR6-negative mice have an attenuated inflammatory response to cigarette smoke (Bracke et al. 2006, Bracke et al. 2007), emphasising the role of chemokines in cigarette smoke-induced lung injury.

IL-6 was the only proinflammatory cytokine with significantly lower mRNA expression in smokers than in non-smokers in our study. Supporting our results, the IL-6 protein content of the BALF has been shown to be lower in smokers than in non-smokers (McCrea et al. 1994), although contradictory results have again been published (Kuschner et al. 1996). Kotani et al. (Kotani et al. 2000) found no difference in the IL-6 mRNA expression of alveolar macrophages under anaesthesia.

Our results demonstrated no difference in TNF- α mRNA expression in BAL cells between smokers and non-smokers. TNF- α has been extensively investigated in COPD. In earlier studies, TNF- α has been found to be increased in the sputum and plasma of COPD patients compared to healthy subjects (Keatings et al. 1996, Takabatake et al. 2000). TNF- α levels have also been shown to be increased in the plasma of healthy smokers compared to non-smokers (Petrescu et al. 2010). Furthermore, TNF- α receptor polymorphisms have been suggested in the pathogenesis of COPD (Molfini 2004). Cigarette smoke has been shown to elicit a greater response in TNF- α expression in the bronchial epithelial cells of COPD patients and non-smokers than in those of asymptomatic smokers (Mills et al. 1999). Conversely, in a study by Lim et al. (2000), alveolar macrophages of smokers released more TNF- α than those of non-smokers. Inflammation and tissue damage caused by CS is inhibited in TNF- α -deficient mice (Churg et al. 2002). Interestingly, no studies showing elevated concentrations of TNF- α in the BALF of COPD patients or smokers could be found.

Increased expression of CXCL8 in the airways is one of the most consistent findings in COPD (Chung 2001). In our study, CXC chemokine CXCL8 showed a tendency towards lowered expression in smokers compared to non-smokers, but the difference was not significant. This is consistent with previous results of BALF protein expression showing no change or decreased expression of CXCL8 in BALF in smokers compared to non-smokers (McCrea et al. 1994, Tanino et al. 2002) but contradictory results have also been published (Mio et al. 1997, Ohta et al. 1998). However, stimulated and unstimulated alveolar macrophages of smokers have been shown to secrete less CXCL8 than those of non-smokers or COPD patients (Culpitt et al. 2003, Ohta et al. 1998, Tanino et al. 2002). Most of our cells were macrophages and we examined mRNA in cells, not the protein content in BALF. Kotani et al. (2000) also found no difference in the mRNA expression of CXCL8 in BAL cells between smokers and non-smokers in patients under anaesthesia. CS stimulates CXCL8 release from bronchial epithelial cells *in vitro* in non-smokers and in COPD patients, but not in asymptomatic smokers (Mills et al. 1999, Mio et al. 1997). Thus, it seems that elevated expression of CXCL8 in the alveolar space and cells is related more to clinical disease progression than to the effects of long-term cigarette smoke exposure *in vivo*.

In our study, healthy smokers showed a significant decrease in the expression of Th1 cytokine IFN- γ and Th2 cytokine IL-13 mRNA compared to healthy non-smokers. However, the difference in the expression of mRNA for IFN- γ was probably not biologically relevant (Table 7). Moreover, mRNA expression of IL-13 was quite low. In a mouse model, IL-13 and IFN- γ have been observed to cause emphysema to some extent through matrix metalloproteinase and cathepsin-mediated mechanisms, also making them candidate mediators in COPD (Wang et al. 2000, Zheng et al. 2000).

Expression of TNF- β was also lower in smokers than in non-smokers. TNF- β /lymphotoxin α is a member of the TNF superfamily and a Th1-type cytokine secreted by lymphocytes (Aggarwal 2003). It is structurally very similar to TNF- α but has functionally different properties, although both act via the same receptors TNFR1 and TNFR2. TNF- β has been much less studied than TNF- α . Polymorphisms of the TNF- β gene have been linked to various inflammatory diseases, but not to asthma or COPD.

Table 7. mRNA expression of cytokines and chemokines in BAL cells from smokers

| | | <i>Never smokers (N = 8)</i> | <i>Asymptomatic smokers (N = 5)</i> | <i>p-value</i> |
|---------------------------|--------------------------------|--------------------------------------|---|----------------|
| Proinflammatory cytokines | IL-1 α | 979.1 \pm 126.6 | 745.5 \pm 236 | 0.106 |
| | IL-1 β | 1240.5 \pm 222.3 | 895.5 \pm 315.2 | 0.127 |
| | IL-6 | 65.2 \pm 11.3 | 11.4 \pm 4.3 | 0.003 |
| Th1 cytokines | TNF- α | 12.5 \pm 4.9 | 3.8 \pm 2.3 | 0.284 |
| | IL-2 | 2.9 \pm 0.7 | 1.1 \pm 0.2 bd | 0.03 |
| | IL-12p35 | 3.3 \pm 0.8 | 1.6 \pm 0.4 bd | 0.171 |
| | IL-12p40 | 2.5 \pm 0.4 | 1.1 \pm 0.2 bd | 0.011 |
| | IL-15 | 35.2 \pm 6.4 | 43.2 \pm 24.9 | 0.435 |
| | IL-16 | 38.2 \pm 14.8 | 37.7 \pm 18.1 | 0.943 |
| | IL-18 | 973.4 \pm 149.1 | 858 \pm 305.9 | 0.435 |
| | TNF-β | 79.6 \pm 20.8 | 12.8 \pm 5.3 | 0.006 |
| Th2 cytokines | IFN-γ | 610.9 \pm 104.7 | 503.7 \pm 266.9 | 0.045 |
| | IL-4 | 1.7 \pm 0.4 | 1.1 \pm 0.2 bd | 0.284 |
| | IL-5 | 2.1 \pm 0.7 | 1.1 \pm 0.2 bd | 0.171 |
| | IL-10 | 22.4 \pm 9 | 20.9 \pm 10.3 | 1 |
| | IL-13 | 6.3 \pm 1.7 | 2.2 \pm 0.9 | 0.03 |
| Chemokines | IL-8 | 1093.7 \pm 379 | 472.3 \pm 100.1 | 0.435 |
| | CCL11 | 15 \pm 11.2 | 3.3 \pm 0.7 | 0.284 |
| | CCL5 | 5340 \pm 1998 | 92.5 \pm 26.4 | 0.002 |
| | CCL20 | 216.5 \pm 63.6 | 13.7 \pm 4.3 | 0.002 |
| | CCL22 | 333.6 \pm 153.2 | 171.6 \pm 69.8 | 0.222 |
| | CCL4 | 1682.4 \pm 380.4 | 146.8 \pm 49.4 | 0.003 |
| | CCL3 | 4255.7 \pm 2110 | 653.7 \pm 197.6 | 0.006 |

bd = Borderline of detection. Expression is reported as relative expression units.

No influence on inflammatory cells in biopsies

In the bronchial biopsy specimens, no significant differences were observed between the smokers and the non-smokers in the differential cell counts in the lamina propria, as in the study by Lams et al. (2000). A similar bronchoscopy study but with a higher number of patients by Amin et al. (2003) revealed elevated numbers of macrophages, neutrophils, eosinophils and mast cells in biopsies in asymptomatic smokers in comparison with never smokers. Subjects in the study by Lams et al. had better lung function than in our study or in that of Amin et al. Additionally, the small number and younger age of subjects in our study, and the section area used in cell calculations may explain the differences. Elevated inflammatory cell counts in bronchial biopsies or lung resections are a consistent finding from patients with COPD or chronic bronchitis (Di Stefano et al. 1998, Lams et al. 2000, O'Shaughnessy et al. 1997, Saetta et al. 1993). Inflammatory cells, especially CD8+ cells, are often inversely correlated with the FEV1 as a percentage of the predicted in all smokers (Di Stefano et al. 1998, Ekberg-Jansson et al. 2000, Isajevs et al. 2011, Lams et al. 2000, O'Shaughnessy et al. 1997). Larger and smaller airways react differently to smoking (Battaglia et al. 2007, Isajevs et al. 2011). Unfortunately, the lung resection samples used to examine changes in the small airways were from patients operated to remove various lung tumours, which are sometimes not defined more precisely. There is evidence that lung cancer modifies the inflammation in the airways (Hoser et al. 2003, Matanic et al. 2003). Furthermore, if a tumour is present

in one lung, the other is also affected. Isajevs et al (2011) also recently detected increased numbers of inflammatory cells in larger airways from smokers in comparison to non-smokers, but the patients were operated for lung pathology. Studies on lung resection samples have additionally shown inflammation in the small airways in smokers without COPD. It has been reported that there are elevated numbers of macrophages, neutrophils, activated and total eosinophils as well as a decreased CD4+/CD8+ T-cell ratio in the submucosa of the smaller airways in smokers compared to non-smokers (Isajevs et al. 2011, Lams et al. 1998). As COPD is a disease of the smaller airways, the changes in the periphery of the lungs may be more important, but also more difficult to determine.

4.1.2 Diisocyanate-induced asthma (II)

The long-term prognosis of occupational DIA is not very good. Studies on inflammation in the airway are sparse. Thus, we examined biopsies and BAL in relation to clinical parameters in a 2- to 3-year follow-up. In addition to the cessation of exposure to DIIs, patients used ICS treatment throughout the follow-up. The second and third study visits were a mean of 7.9 and 33.5 months after the first study visit, respectively. We lost 5 of 17 patients during the follow-up. The histamine challenge test was lacking from two patients at the second visit and from one patient at the third visit. In addition, a couple of mRNA samples and biopsies were discarded because of poor quality. There was a significant decline in the absolute forced vital capacity (FVC) of 300 ml ($p = 0.015$), but not in FVC as a percentage of the predicted value during the follow up. There was also an almost significant decline in FEV1 of 290 ml ($p = 0.023$, Bonferroni-corrected level of significance $p < 0.02$), but not in FEV1 as a percentage of the predicted value. Declining lung function, despite treatment and the cessation of exposure, has also been reported in previous studies (Padoan et al. 2003, Piirila et al. 2000, Pisati et al. 2007). The annual decrease in lung function of DIA patients in our study was greater than that reported for smokers (Fletcher and Peto 1977, Lee and Fry 2010). The decrease was even less in smokers than non-smokers. Whether this was a coincidence or actually related to biology remains undetermined in this study. One explanation might be that smokers gained additional benefit from ICS (Pauwels et al. 1999). DIIs are irritative, and even short exposure to low concentrations can cause measurable changes in airway function (Vandenplas et al. 1999). Thus, the long-term follow-up results are not very surprising, even though all the patients were receiving treatment.

Elevated number of IL-4-positive cells and increasing numbers of macrophages in the lamina propria

Eosinophils were slightly elevated from 0.5% to 1% in patients with DIA in the BALF compared to controls at the first visit ($p = 0.012$, Bonferroni corrected level of significance $p < 0.01$), with no difference in other cell lines or total cells. Previous research has also shown a slight increase in BAL eosinophils in DIA (Fabbri et al. 1987, Paggiaro et al. 1990).

The total number of cells and percentage of lymphocytes decreased by a half between the first and the second examinations during the ICS treatment.

The number of macrophages in the biopsies was significantly higher in DIA patients in the second (n = 9) and third (n = 7) examinations than in controls (n = 14). Elevated numbers of mononuclear cells have been found in a previous study under the epithelium, despite the cessation of exposure (Saetta et al. 1992). At least HDI modifies the morphology and gene expression of peripheral blood mononuclear cells (PBMC), which implies a role for mononuclear cells in the pathogenesis of DIA even without adaptive immunity (Wisnewski et al. 2008). Patients with DIA (n = 12) had more IL-4-secreting cells in the lamina propria than controls (n = 13) at the first and second study visits. This is consistent with previous results (Maestrelli et al. 1997). The number of IL-4-positive cells decreased nonsignificantly on follow-up while patients were on ICS, and we found no difference in IL-4-positive cells between controls and patients (n = 9) in the third examination. IL-4-positive cells correlated with NSBHR almost significantly in the second examination ($\rho = -0.56$, $p = 0.058$), but in the third examination (n = 6) NSBHR was strongly correlated with CD3+ ($\rho = -0.88$, $p = 0.021$) and CD4+ ($\rho = -0.94$, $p = 0.005$) cells. In a study by Jones et al. (2006), no increase in IL-4 mRNA was observed after acute active challenge with DIA *in situ* in bronchial biopsies from patients exposed predominantly to HDI. However, IL-5 mRNA was increased in comparison to control challenge accompanied by a nonsignificant increase in CD4+ cells, eosinophils and neutrophil elastase staining in their study. CD4+ cells were also increased in comparison to controls. As in our study, the numbers of DI-specific IgE-positive patients were quite low. In our study, bronchoscopy was performed at the first visit 48 h after the challenge, as opposed to 24 h in the study by Jones et al. (2006). Although the timing of the bronchoscopy was different in our study, the possible effect of specific challenge testing on the inflammatory cell numbers in the first examination cannot be excluded. Interestingly, in the study by Jones et al. (2006), patients with both negative and positive specific inhalation challenge test results mounted similar inflammation in response to active challenge. In the same study, an increase in cells positive for IL-4 mRNA was seen in other atopic asthmatics after specific challenge for their allergens, in contrast to DIA patients. Protein expression was not studied, which might explain the differing results. No data on ICS use was provided, either.

Persistent NSBHR is associated with elevated expression of mRNA for TNF- α , IL-6 and IL-15 in BAL cells

In DIA patients, NSBHR decreased during the follow-up in accordance with previous results (Padoan et al. 2003, Piirila et al. 2000, Pisati et al. 2007). However, consistently with previous studies, 5 out of 11 patients still had NSBHR at the third examination. Hyperreactive patients displayed a higher expression of TNF- α ($p = 0.017$), IL-6 ($p = 0.014$) and IL-15 ($p = 0.012$) mRNA in BAL cells than non-hyperreactive ones. These patients were on ICS using budesonide 800-1600 $\mu\text{g}/\text{d}$ or fluticasone 1000 $\mu\text{g}/\text{d}$. None of the hyperreactive patients were smokers.

TNF- α , IL-6 and IL-15 have been implicated in the pathogenesis of allergic asthma, and TNF- α and IL-6 additionally in DIA. TDI has been shown to induce IL-6 expression in human bronchial epithelial cells (Mattoli et al. 1990), although TNF- α was not examined in that study. The lack of TNF- α attenuated Th2 inflammation and airway hyperreactivity in a mouse model of TDI-induced DIA (Matheson et al. 2002). TNF- α also has an impact on glucocorticoid responsiveness, which is interesting, since all our patients with DIA were using ICS. In the study of Franchimont et al. (1999), pretreatment of human whole-blood cell cultures with TNF- α inhibited the suppression of LPS-induced IL-6 secretion by dexamethasone. This was mediated through down-regulation of glucocorticoid receptors (GR) on monocytes. Subsequently, the pathways and interactions of TNF- α and GRs leading to resistance to glucocorticoids have been revealed in great detail (Van Bogaert et al. 2010). Patients still hyperreactive at the end of the follow-up in this study may be classified as at least having slightly glucocorticoid-resistant asthma. At least five out of seventeen enrolled patients with DIA fitted this category, further emphasising the poor prognosis. In our study, cytokines were quantified in BAL cells, which were mainly macrophages. PBMCs from DIA patients have been shown to secrete more TNF- α *in vitro* under antigen challenge (Lummus et al. 1998). Most of those patients were exposed to HDI. In human patients with mild asthma, TNF- α inhalation elicits increased NSBHR to metacholine along with increased expression of cytokines, including IL-15 (Thomas and Heywood 2002). IL-15 inhibits apoptosis of eosinophils by stimulating the expression of the powerful eosinophil survival factor granulocyte-macrophage colony stimulating factor (GM-CSF) (Hoontrakoon et al. 2002). The exact role of eosinophils, even in human atopic asthma, is still open after decades of research, but they appear to have a role in the pathogenesis of airway hyperreactivity, among other effects, at least in severe eosinophilic asthma (Rothenberg and Hogan 2006). In accordance with this, IL-15 antibodies were shown to abrogate OVA-induced airway hyperreactivity and allergic inflammation in Balb/c mice (Ruckert et al. 2005). TNF- α might be the key to hyperreactivity in our DIA patients, although this needs to be confirmed. TNF- α inhibitors might provide help for these patients.

We found no difference in the expression of the studied cytokines and chemokines between DIA patients and controls in any examination. Neither was there any change in inflammatory mediators between visits. When all patients were analysed together, cytokines and chemokines did not correlate with NSBHR or the results of other pulmonary function tests. The specific challenge testing might have influenced the mRNA expression in the first examination, as shown by Jones et al. (2006). However, as the results for patients were similar to those for controls, this seems unlikely.

4.1.3 Mouse model of allergic asthma (III)

WT mice

First, we aimed to demonstrate that, as expected, OVA induces allergic inflammation in the airways in WT Balb/c mice. OVA-sensitized mice exhibited vigorous lung eosinophilia, a strong Th2-type cytokine response, and increased airway reactivity to inhaled Mch. A significant and prominent influx of eosinophils and lymphocytes in the BAL was observed in OVA-challenged mice. The mRNA levels of proinflammatory cytokines (TNF- α , IL-6, IL-1 β), Th2 cytokines (IL4, IL5, IL13) and CCL11 were significantly increased in the lung OVA-challenged mice compared to PBS-challenged mice with modestly increased expression of IFN- γ . Haematoxylin-eosin stained lung samples exhibited collections of inflammatory cells. NSBHR to inhaled Mch was significantly increased in the OVA-challenged mice group compared to PBS-challenged controls measured as Penh. The main results are summarised in Table 7 and Figure 1.

KO mice

STAT4^{-/-} and STAT6^{-/-} mice had attenuated inflammation in response to OVA, with similarities and differences. Cell calculations in biopsies were not carried out, but the influx of eosinophils and lymphocytes in BAL was suppressed in KO mice. The number of eosinophils was especially low in STAT6^{-/-} mice and that of lymphocytes in STAT4^{-/-} mice. In STAT6^{-/-} mice, more neutrophils were instead seen. STAT4^{-/-} animals were similarly or more hyperreactive compared to WT mice, while STAT6^{-/-} mice showed attenuated hyperreactivity. The main results are summarised in Table 8 and Figure 1.

Table 8. BALF cell numbers of PBS- and OVA-challenged mice.

| | WT | | STAT4 ^{-/-} | | STAT6 ^{-/-} | |
|-----------------------------------|--------------------|---------------------------|----------------------|--------------------------|----------------------|--------------------------|
| | PBS (n=8) | OVA (n=8) | PBS (n=8) | OVA (n=9) | PBS (n=8) | OVA (n=8) |
| Eosinophils (million/l) | 0.2 ± 0.36 | 105.35 ± 27.43 *** | 0.28 ± 0.33 | 80.97 ± 32.79 *** | 0.03 ± 0.09 | 1.45 ± 1.31 ** |
| Lymphocytes (million/l) | 0.09 ± 0.16 | 18.14 ± 5.75 ** | 0.2 ± 0.35 | 1.59 ± 1.07 ** | 0.02 ± 0.04 | 3.37 ± 1.64 *** |
| Macrophages (million/l) | 20.02 ± 0.57 | 22.5 ± 9.8 | 60.34 ± 21.32 | 25.89 ± 15.42 * | 22.05 ± 6.83 | 24.46 ± 11.59 |
| Neutrophils (million/l) | 0.2 ± 0.35 | 2.79 ± 2.11 ** | 2.14 ± 0.71 | 6.09 ± 3.14 ** | 0.47 ± 0.44 | 38.29 ± 14.61 *** |

Comparisons were made between PBS- and OVA-challenged mice within the WT, STAT4^{-/-} and STAT6^{-/-} groups. *p < 0.05, **p < 0.01, ***p < 0.001.

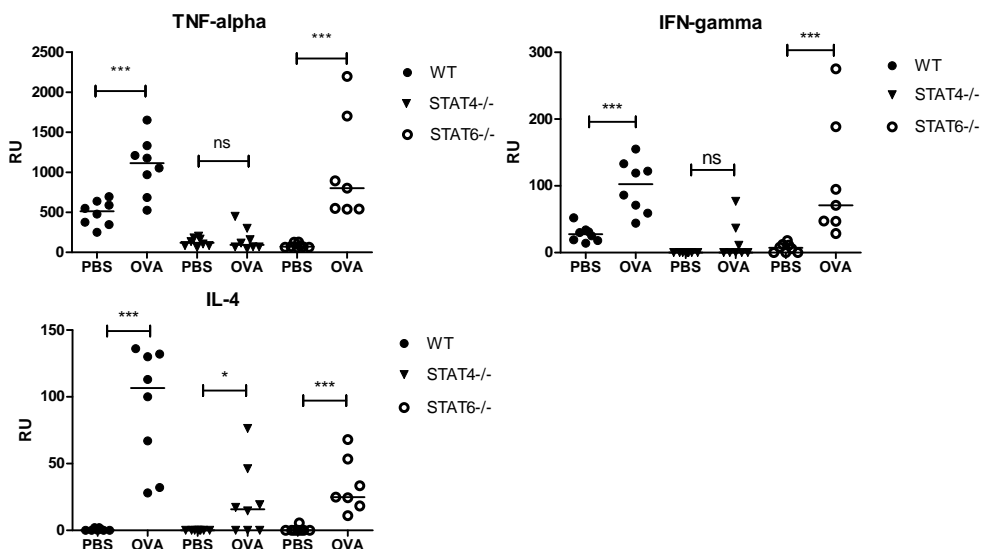


Figure 1. Expression of mRNA for cytokines in PBS- and OVA-challenged WT, STAT4^{-/-} and STAT6^{-/-} mice. RU = relative expression unit. *p < 0.05, **p < 0.01, ***p < 0.001

In a recent study by Chapoval et al. (2011), some interesting remarks were made on inflammation and NSBHR in STAT6^{-/-} mice. Their Penh results showed higher NSBHR to Mch in PBS-challenged control STAT6^{-/-} mice than in OVA-challenged WT mice, suggesting naïve hyperreactivity in STAT6^{-/-} mice. The difference was quite clear. Their experimental protocol was similar enough to ours, although the number of mice was almost one-half lower. We performed the same analysis. We found a difference at one concentration point of Mch, but the groups were otherwise similar (data not shown). It is a general view that when conclusions are drawn on changes in NSBHR, they should be confirmed with another method than WBP. Changes in WBP might simply reflect variation in the respiratory pattern caused by metacholine (*see* Shortcomings of the substudies section). Chapoval et al. (2011) found no difference in NSBHR between OVA- and PBS-challenged STAT6^{-/-} mice, similarly to our results.

Interestingly, both STAT4^{-/-} and STAT6^{-/-} mice exhibited an attenuated response to OVA as measured in mRNA expression of Th2 cytokines and CCL11 in lung tissue. For STAT6^{-/-} this is not surprising. However, allergen-challenged STAT4^{-/-} mice have shown decreased expression of chemokines, not Th2 cytokines, in BALF (Kim et al. 2010b, Raman et al. 2003). We studied mRNA expression in lung tissue, which might explain the difference. The most striking difference between STAT4^{-/-} and STAT6^{-/-} mice was the absence of OVA-stimulated TNF- α and IFN- γ expression in STAT4^{-/-} mice. As STAT4 induces the expression of IFN- γ and the Th1 phenotype of CD4⁺ cells (Pfitzner et al. 2004), was this an expected result. Th1 cells are able to induce allergic airway inflammation and NSBHR in mice under suitable conditions after OVA challenge (Hayashi et al. 2007). These wide disturbances in the allergic inflammation of STAT4^{-/-} mice raise interest in the role of STAT4 in allergic airway inflammation.

4.2 Expression of tenascin-C

4.2.1 WT mice (III)

First, we examined whether the expression of tenascin-C changed after OVA challenge. Tenascin-C mRNA levels were significantly increased in the lung tissue of OVA-sensitized WT mice compared to controls. In the immunohistological analysis, tenascin-C was strongly up-regulated in the alveolar BM as small spots throughout the parenchyma of OVA-challenged mice. Staining of the bronchial basement membrane with tenascin-C was negative in both groups. In patients with asthma the expression of tenascin-C is increased in the bronchial BM, which was not seen in this OVA-induced model of allergic airway inflammation (Laitinen et al. 1997, Nakahara et al. 2006). In our study, TGF- β 1 mRNA levels were similar in OVA- and PBS-challenged mice (unpublished). Although the importance and over-expression of TGF- β 1 is well established in asthma under provocation, and it is known to stimulate the expression of tenascin-C in the airways (Bosse and Rola-Pleszczynski 2007, Doerner and Zuraw 2009), an increase in tenascin-C and TGF- β 2, but not TGF- β 1, has also been seen under the epithelium in atopic human beings after allergen challenge (Torrego et al. 2007). Furthermore, numerous other mediators for increased expression of tenascin-C are readily available in the airways of OVA-challenged mice such as IL-4, IL-13, TNF- α and IFN- γ (Flood-Page et al. 2003a, Harkonen et al. 1995, Makhluף et al. 1996, Nakahara et al. 2006, Rettig et al. 1994, Syed et al. 2005).

4.2.2 STAT4^{-/-} and STAT6^{-/-} mice (III)

We next aimed to examine whether tenascin-C expression changed in KO mice with defects in Th1 (STAT4^{-/-}) and Th2-type responses (STAT6^{-/-}) to find *in vivo* regulators of tenascin-C expression. This was the first time that the regulation of tenascin-C expression has been studied using STAT4 and STAT6 KO animals. Most mediators of tenascin-C expression have been found *in vitro*. To our surprise, the expression of tenascin-C in STAT6^{-/-} mice was quite similar to that of WT mice, although nonsignificant suppression of immunoreactivity was seen in semiquantitative analysis. In contrast, the attenuated mRNA expression and immunoreactivity of tenascin-C in STAT4^{-/-} mice was clear. As the most important factor separating OVA-challenged STAT4^{-/-} and STAT6^{-/-} mice was the low expression of TNF- α and IFN- γ in STAT4^{-/-} animals, we examined whether these cytokines stimulated tenascin-C expression from human dermal fibroblasts and foetal lung fibroblasts. TNF- α is known to induce tenascin-C production in both lung fibroblasts and bronchial epithelial cells (Harkonen et al. 1995, Nakamura et al. 2004). Furthermore, TNF- α and IFN- γ have been shown to induce tenascin-C expression synergistically with cyclic strain in interstitial cells from aortic valves spoiled by rheumatic fever (Jiang et al. 2009). Foetal MRC-9 showed a nearly 3-fold increase in the expression of tenascin-C mRNA in response to TNF- α . Because these were

foetal cells and tenascin-C is mostly a foetal protein, we also wanted to demonstrate the same results with cells from adult tissues and with IFN- γ from human dermal fibroblasts. As suspected, the increase in expression was not as clear as with foetal cells, but significantly increased expression (determined as at least a 2-fold increase) was seen with TNF- α and TNF- α + IFN- γ after 6 h. Some synergism was seen. Protein expression was determined after 18 hours of incubation with the combination and showed an increase. Incubation with IFN- γ alone elicited much weaker mRNA expression of tenascin-C than the combination of TNF- α and IFN- γ .

4.2.3 DIA (unpublished data)

To examine whether tenascin-C overexpression in BM is also a feature in DIA in comparison to controls, we measured the thickness of the tenascin-C layer in the bronchial BM. The tenascin-C layer of the bronchial membrane was measured from paraffin embedded biopsies as a small preliminary study to determine whether DIA patients have similar tenascin-C expression to atopic or other non-atopic patients (Karjalainen et al. 2003, Laitinen et al. 1997). The study began in the 1990s, and some of the frozen samples were too old to be analysed, as measurement of tenascin-C was not included in the original study design. Furthermore, the paraffin-embedded samples were often of better quality and the morphology was also better. Unfortunately, paraffin sections were available for only 13 patients and mostly from one examination, which varied. Thus, all the 19 samples from 13 patients were compared with the controls. Including only a particular visit would have considerably reduced the number of available samples for analysis.

Most of the controls were only faintly reactive. All the strong reactivity was found among DIA patients. We found a slight difference between DIA patients and controls in the thickness of tenascin-C layer (Figure 2). However, the mean thickness in DIA patients was 2.8 μm when in chronic asthma and newly diagnosed non-atopic asthma it has been shown to be 8 μm and 6.3 μm , respectively (Karjalainen et al. 2003, Laitinen et al. 1997). None of the DIA patients had a tenascin-C layer more than 6 μm in thickness. Although different antibodies were used, the mean thickness of the tenascin-C layer in the BM of controls was at a similar level as detected in a previous study (Laitinen et al. 1997). When the intensity of the immunoreactivity was semiquantitatively assessed, no difference was detected between the patients and the controls. Furthermore, there was no difference between the visits or hyperreactive and non-hyperreactive patients. Thus, according to our results, tenascin-C is not a very good marker in DIA.

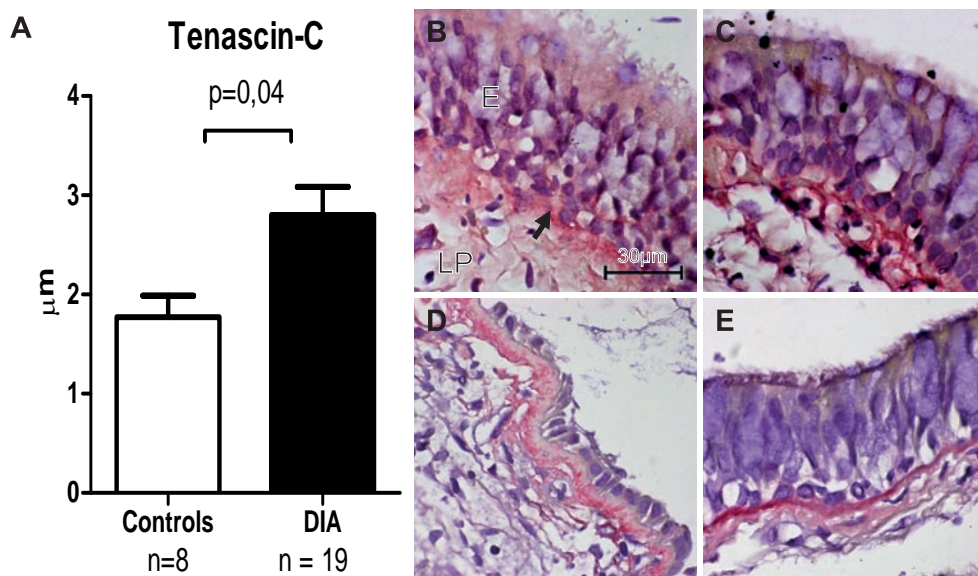


Figure 2. Immunoreactivity of tenascin-C in DIA patients. A. The thickness of tenascin-C immunoreactivity in the bronchial basement membrane. Results are expressed as mean \pm SEM. μm = micrometre. B–E. Tenascin-C visualised with APAAP in paraffin-embedded sections. Non-smoking (B) and smoking (C) control subjects had a thin bronchial basement membrane (arrow) with varying tenascin-C reactivity. In contrast, DIA patients had a thick basement membrane at the beginning of the follow-up (D), also with varying tenascin-C reactivity. The thickness of the bronchial basement membrane decreased during the follow-up and treatment with ICS in some patients (E). D and E are from same patient. E = epithelium, LP = lamina propria.

4.3 Expression of laminin $\alpha 2$ and $\beta 2$ chains

4.3.1 Mice (unpublished data)

In addition to tenascin-C, we examined the expression of laminin $\alpha 2$ and $\beta 2$ chains in acute allergic airway inflammation in mice. We aimed to relate the changes to human asthma and possibly find regulators of laminin expression. Laminin $\alpha 2$ and $\beta 2$ were selected because of our previous results in human asthma patients (Altraja et al. 1996). Expression of laminin $\alpha 2$ and $\beta 2$ mRNA was significantly decreased in OVA-sensitized WT mice (Figure 3A). However, no difference was found in immunoreactivity between the OVA- and PBS-challenged mice. Moreover, no change was seen in laminin expression between the OVA- and PBS-challenged STAT4 $^{-/-}$ and STAT6 $^{-/-}$ mice (data not shown) in mRNA or immunoreactivity. The staining pattern was similar to WT mice.

Immunoreactivity of laminin $\alpha 2$ and $\beta 2$ chains in the lung was similar to that reported in earlier studies (Sasaki et al. 2002, Sorokin et al. 1997). Laminin $\beta 2$ was detected in various BMs, including alveolar structures (Figure 3B and C). It was expressed in the BM under

the epithelium, and in the BM surrounding the bronchial smooth muscle in the bronchi as well as in the bronchioles. In the large arterioles and veins it was evident in several layers. Mesenchymes were seen as completely unreactive areas around the blood vessels and the bronchi. Laminin $\alpha 2$ (Figure 3C and D) was only found in some alveolar structures and bronchial BM. Some immunoreactivity for the laminin $\alpha 2$ chain was detected in the bronchial smooth muscle. It was not expressed in the innermost layer of the arterioles, but stained the arterial smooth muscle similarly to the staining pattern of the laminin $\beta 2$ chain.

We studied quite acute allergic airway inflammation, and remodelling is more related to chronic models, which might explain why no changes were seen in laminin expression in immunohistochemistry, while mRNA expression was decreased. Furthermore, the laminin expression pattern in mice markedly differs from that in humans, which makes it impossible to study the appearance of the laminin $\alpha 2$ chain in asthma, because it already is expressed in the bronchial BM. In asthma patients, laminin $\alpha 2$ is expressed in the bronchial BM, while controls are negative (Altraja et al. 1996). The amount of laminin protein expression might be worth examining in the mouse models of asthma, but based on our preliminary results in RT-PCR and immunohistochemistry we assumed that it would not give any new information, at least in this mouse model. In healthy humans, laminin $\beta 2$ expression in the lung and especially in the bronchial BM is also minor compared to that in mice (Altraja et al. 1996). Increased expression of laminin 111 in the mouse lung after OVA challenge has previously been demonstrated (Christie et al. 2004).

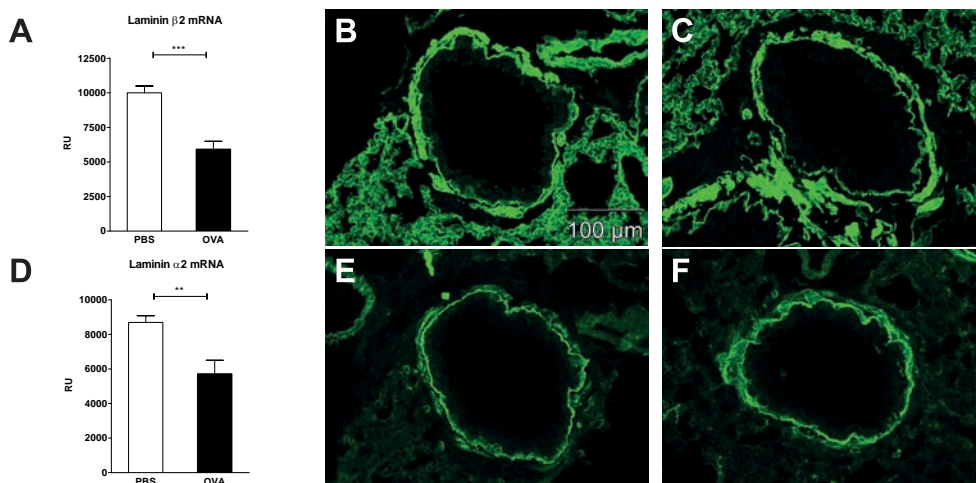


Figure 3. Laminin $\alpha 2$ chain and laminin $\beta 2$ chain expression in wildtype mice. The relative mRNA expression of laminin $\alpha 2$ (D) and $\beta 2$ (A) chains was lower in the lung of ovalbumin-challenged than control mice. Mouse lung immunoreactivity to laminin $\beta 2$ in the controls (B) and in the OVA-challenged mice (C), as well as to laminin $\alpha 2$ (E and F respectively), was similar. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 8$ mice/group RU = relative expression unit. Results are expressed as mean \pm SEM.

4.3.2 Patients (IV)

We hypothesised that patients with diagnosed asthma might have increased co-expression of laminin $\alpha 2$ and $\beta 2$ chains in comparison to patients with asthma-like symptoms and controls, and that the laminin $\alpha 2$ chain would mostly be seen in patients with asthma. Biopsies of sufficient quality were available for all 8 controls, 26/35 patients with asthma for less than 2 years, and 12/15 patients with asthma symptoms. The bronchial BM of all patients with asthma and asthma symptoms was positive for the laminin $\beta 2$ chain. In contrast, 6 out of 8 controls were negative. Immunoreactivity was seen as a more or less continuous line under the epithelium. Laminin $\alpha 2$ chain was expressed in the bronchial BM in only one patient with asthma symptoms, 14 out of 26 patients with asthma and none of the controls. The immunoreactivity for laminin $\alpha 2$ was present as a fragmented line under the epithelium. Electron microscopy and double stainings were not performed, but the expression was at the upper margin of the BM, which corresponds to the lamina densa. The laminin $\alpha 2$ and $\beta 2$ chains were co-expressed in the same patients who had immunoreactivity for the $\alpha 2$ chain. We found no difference in the expression patterns of $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 3$, $\gamma 1$ or $\gamma 2$. The thickness of the laminin-positive layer for these normally expressed chains was not defined, because we were looking for differences in spatial chain expression. These results confirmed our hypothesis that the laminin expression pattern differentiated patients with diagnosed asthma from those with asthma-like symptoms and from controls.

The results strengthened our previous view that the expression of the laminin $\beta 2$ chain is followed by laminin $\alpha 2$ chain expression in asthma. This sequence is the reverse of that seen during development (Altraja et al. 1996, Virtanen et al. 1996). Longitudinal studies are needed to clarify the role of laminin $\alpha 2$ and $\beta 2$ in the development of asthma. As laminins are most probably secreted as heterotrimers, laminin121 is replaced by laminin 221. As the change occurs in the BM and not in ASM, this probably does not affect the submucosa. However, laminin $\alpha 2$ in the BM might be a good substrate for adhesion of actively secreting myofibroblasts (Tran et al. 2006), thus directly influencing inflammation and further remodelling. Moreover, LTD4 has been shown to increase laminin $\beta 2$ expression in bronchial epithelial cells, connecting the increased expression directly to airway inflammation in asthma (Altraja et al. 2008).

In contrast to our results, Amin et al. (Amin et al. 2005) showed immunoreactivity for the laminin $\alpha 2$ chain in and stronger reactivity for the $\beta 2$ chain the bronchial BM in healthy controls than in the present study. All their articles on the thickness of the laminin and tenascin-C layers have shown at least two times thicker immunoreactivities than our studies (Amin et al. 2000, Amin et al. 2005). In our studies we have tested different planimetric methods and obtained comparable results not depending on the method (Autocad/planimetric table and ImageJ/computer screen planimetry). At least 4–5 researchers in our group have gained similar results on the thickness of tenascin-C layer in the bronchial BM of controls

in different studies. Furthermore, considering the scales, it is very difficult to believe that the mean thickness of the tenascin-C layer would be over 15 μm in asthmatics and over 10 μm in controls (Amin et al. 2000). Moreover, in their laminin publication, the photograph of laminin $\gamma 2$ showed almost a fragmented line and somehow the mean thickness was still 2 μm in controls (Amin et al. 2005). This may naturally be due to selection of certain areas in the picture to highlight the result.

5. General discussion

5.1 *Why are macrophages inactive in smokers?*

Macrophages appeared very quiescent in the airway lumen in our study, despite the vast numbers of them gathering in the airways of smokers. Without a specialised immune response, the development of adaptive immunity would be impaired. This may explain the recurring respiratory infections of smokers. As macrophages are the key link between innate and adaptive immunity as well as being able to sequester MMPs directly, causing tissue damage, this might be one mechanism to control the inflammation and protect the tissue from the destruction seen in COPD (Tetley 2002). Mills et al.(1999) hypothesized that the lowered cytokine release of cells from smokers compared to non-smokers and COPD patients could be a defence mechanism against the ongoing inflammation and development of COPD. It has been proposed during the past few years that COPD could be an autoimmune disease (Cosio and Agusti 2010). Smoking might be just one of the triggers or predisposing factors. These hypotheses need to be tested with further studies, but might explain why the reactions of the cells differ between subjects and cell lines (Smith et al. 2010). Rather than comparing smokers with and without COPD at one time point, there is a need for longitudinal studies where individuals are studied at multiple time points during the course of the disease.

5.2 *Mice models: do we need them?*

We used a mouse model and knockouts to study the expression of tenascin-C in allergic airway inflammation. Animal models have been a hot topic in meetings on respiratory medicine during recent years. They are used in studying the underlying mechanisms of diseases. Most of the pathogenetic mechanisms of asthma have been revealed using animal models (Kips and Pauwels 1999, Takeda and Gelfand 2009). Cell cultures are much too simplified to be used alone. However, animal models are not without problems. First, the best model for a given question should be found to mimic the responses in human. No animal model so far has precisely replicated the features of human asthma and probably never will. For example, chronic exposure models with OVA would be better for studying remodelling (Kumar et al. 2008), but what phase of human asthma does, for instance, 8 weeks of sensitisation represent? Mice do not have spontaneous asthma. Still, much valuable information can be obtained from animal models if the results are interpreted correctly, as reviewed in an article by Finkelman et al.(Finkelman and Wills-Karp 2008). Obtaining the same information on the regulation of tenascin-C expression *in vivo* as in this study would have been very difficult without a mouse model. KO animals are not without problems, either. When one gene is suppressed, others might take over its actions. Furthermore, the time frame when a certain factor is turned on and off might be crucial in the development of an allergic response, and the opposite action might occur at another time point, as shown

with the IL-12-STAT4-IFN- γ pathway (Meyts et al. 2006). As human asthma takes time to develop, the disease is likely to result from very complex networks functioning together. It is not enough to attenuate eosinophilic inflammation or block one cytokine. This is also becoming evident in mouse models of allergic airway inflammation: blocking the action of STAT6 (Foster et al. 2003) might not be enough, and other independent pathways exist (Wills-Karp and Finkelman 2008) that affect the asthma phenotype, such as those involving STAT4 in this study (Kim et al. 2010b).

5.3 Diversity of asthma: DIA

During the years I have studied airway pathology, the diversity of asthma has become very evident (Hargreave and Nair 2009). There is an increasing need for individualised treatment, especially if ICS are not sufficient to control the disease. Asthma is a very common disease. Individualised treatment calls for cheap and non-invasive determination of the problem to target, i.e. phenotyping. Enormous non-targeted trials for new drugs might not be the right way to gain the most benefit from new drugs. We need further studies on the markers related to different forms of asthma. In fact, phenotyping projects are ongoing and already providing new methods to differentiate specific features of different asthma types (Brasier et al. 2010). DIA is a very good example of a diverse form of asthma, as it has common features with allergic and non-allergic asthma, and because of the declining lung function despite ICS, new drugs would be welcome. The sensitizing agent is not a protein, as in classic allergic asthma. One might also expect the pathogenesis to be different. Some have DI-specific IgE while others do not, and as in the case of allergic asthma, not all patients with elevated levels of DI-specific IgE develop reversible airway obstruction (Liu and Wisniewski 2003, Pronk et al. 2007). Those DIA patients still hyperreactive at the end of the follow-up in our study during ICS had very different findings in comparison to those without hyperreactivity, which is very important for further treatment. As DIA is relatively uncommon, co-operation between centres is needed to perform larger studies. Thus the results would be more generally applicable.

5.4 Activation of the developmental mechanisms in asthma

It is not known what induces expression of laminin $\alpha 2$ and $\beta 2$ chains in asthma. The laminin $\alpha 2$ chain was not present in the BM of healthy mature airways at all in this study. Increased expression of the laminin $\beta 2$ chain was already recorded in patients with asthma-like symptoms, and the laminin $\alpha 2$ chain differentiated asthma patients from patients with symptoms but no asthma diagnosis. The expression pattern of laminin $\alpha 2$ and $\beta 2$ chains in the bronchial BM appeared to be reversed in comparison to that occurring during lung development. Our study was cross-sectional. Thus, it was not possible to draw definitive

conclusions on the role of the laminin $\alpha 2$ and $\beta 2$ chains in the development of asthma. Compared to collagen, tenascin-C and fibronectin situated in the lamina reticularis of the bronchial BM, the importance of the laminin $\alpha 2$ and $\beta 2$ chains in the thickening of the airway wall is minor. However, they are an essential part of the EMTU as they are situated in the BM and convey information from the epithelium to the mesenchyma, and vice versa. It might be assumed that the re-expression of these laminin chains is one marker of the reactivated (or unsilenced) developmental genes, which cannot be without consequences (Bousquet et al. 2004), but no functional evidence exists. Considering that the expression of laminins is quite different in mice, as shown in this study and previously, we have one easy and economical method less to study the expression of laminins. Once laminins emerge, it takes time until they disappear, even without any existing external stimuli. Thus, they could be mediators for chronic inflammation and further fibrosis during the “silent phase” of asthma. Tenascin-C is a protein with similar and different properties compared to laminins. It is also important in development, but its expression is much more transient, including in asthma (Kariyawasam et al. 2007). Tenascin-C emerges after allergen challenge and is down-regulated with inflammation, while other remodelling changes remain (Kariyawasam et al. 2007). Thus, tenascin-C behaves more as an inflammatory mediator than a structural protein. As mice have a similar pattern of tenascin-C expression to humans, they are a good species to study the regulation of tenascin-C *in vivo*. The expression of tenascin-C is also strongly regulated by inflammatory mediators, as additionally demonstrated here, and it has been shown to possess many immunomodulatory activities (Jiang et al. 2009, Nakahara et al. 2006, Ruegg et al. 1989, Tucker and Chiquet-Ehrismann 2009). As tenascin-C is already faintly expressed in most controls, an increase in the thickness of the tenascin-C layer in atopic asthma may only reflect the changes in the thickness of the lamina reticularis.

5.5 Shortcomings of the substudies

The major limitation of the first study was the small number of subjects. Performing invasive procedures such as a bronchoscopy with biopsies on healthy subjects must be well reasoned. The number of patients in the subanalysis of the DIA patients was also quite small, mostly attributed to the rarity of the disease. However, biopsy studies on DIA patients have also previously been very restricted in size, and this was among the biggest biopsy studies solely on DIA patients. At least the negative results with such a small number of subjects as we had should be interpreted with caution.

We examined mRNA expression for cytokines and chemokines in the BAL cells or lung tissue in studies I–III without looking into changes in protein expression *in vivo*. Protein is the actual effector. Protein quantification from the BALF is not entirely without problems, either. BAL samples are a mix of a wide variety of substances, especially from smokers. While the BALF from non-smokers is quite clear, the fluid from smokers is a brownish gel.

In vivo proteins are degraded much more easily than mRNA or DNA, and antibody binding might be disturbed, for instance, in ELISA. This can be overcome by isolating the cells of interest and studying the protein expression in primary cell cultures, but then again one has to consider the problems with *ex vivo* studies when interpreting the results. It would have been interesting to examine the mRNA expression of inflammatory mediators in the lung tissue of the patients (study II), but only a few samples were available for mRNA analysis.

It cannot be excluded that the lung function of the smokers had begun to decrease, because FEV1 and FVC1 were significantly lower in smokers than in non-smokers as a percentage of the reference values. However, the smoking subjects cannot be regarded to have developed signs of COPD according to the GOLD standards (GOLD 2008).

The most reliable way to investigate the effects of medication is to plan a blinded randomised placebo-controlled study with enough power. DIA has a poor prognosis, despite the ICS and cessation of exposure. Rendering patients without medication for 6 months to years would have not been possible in this study and would have been unethical. Although the intention was also to examine the effect of corticosteroids in the treatment of DIA, it was impossible to separate the natural progression of the disease from the effects of ICS in our study.

Although there is evidence that Penh is at least to some extent related to changes in airway resistance and bronchoconstriction (Zhang et al. 2009), some have raised concern because there are too many confounding factors (Lundblad et al. 2002) and results should be confirmed with invasive measurements. In this thesis, WBP and Penh were not used to assess the bronchoconstriction *per se*, but to demonstrate, as one parameter among others, that the OVA-induced allergic airway inflammation model is valid. Elevated Penh has also been shown to correlate with invasive measurements of lung function in the FIOH laboratory in this mouse model.

6. Conclusions

Smokers showed higher number of macrophages but decreased cytokine and chemokine mRNA expression in BAL cells compared to non-smokers. This suggests that the smokers have dysfunctional macrophages in the lower airways incapable of producing inflammatory mediators, especially chemokines, that are involved in the activation of other inflammatory cells. This may contribute to the recurring respiratory infections of smokers. Whether this is a protective or harmful effect in relation to other airway pathology is still not known and calls for further studies.

Occupational DIA is a unique type of asthma with a poor prognosis. Patients with NSBHR despite the cessation of exposure and ICS treatment showed increased mRNA expression of mainly macrophage-derived cytokines IL-6, IL-15 and TNF- α in the BAL cells as a sign of ongoing inflammation in comparison to those without hyperreactivity after 2–3 years of follow-up. These might be markers for a poor prognosis and targets for future treatment. Tenascin-C expression was only slightly increased in the bronchial BM in DIA patients. No correlation was found between tenascin-C expression and hyperreactivity. Tenascin-C may not therefore be a useful marker in DIA patients.

In the mouse model of acute allergic airway inflammation, STAT4 $^{-/-}$ mice showed attenuated expression of tenascin-C in the lung, which was related to low expression of IFN- γ and TNF- α . In STAT6 $^{-/-}$ mice with impaired Th2-type immunity, tenascin-C expression was quite similar to WT mice. Furthermore, fibroblasts stimulated by TNF- α and IFN- γ showed increased expression of tenascin-C *in vitro*. Thus, OVA-induced tenascin-C expression was regulated by mediators important in the Th1-type rather than the Th2-type immune response.

We found that the laminin β 2 and α 2 chains are over-expressed very early in the development of asthma in the bronchial BM and are associated with NSBHR. As the laminin α 2 chain was only expressed in patients with asthma, while the expression of laminin β 2 was also more frequent in patients with asthma symptoms than in healthy controls, the laminin α 2 chain may be related to the development of clinical asthma. Longitudinal studies are needed to confirm this. Disturbed expression of laminins is a fine example of remodelling processes taking place in asthmatic airways and reflects the similarities between lung development and remodelling in asthma.

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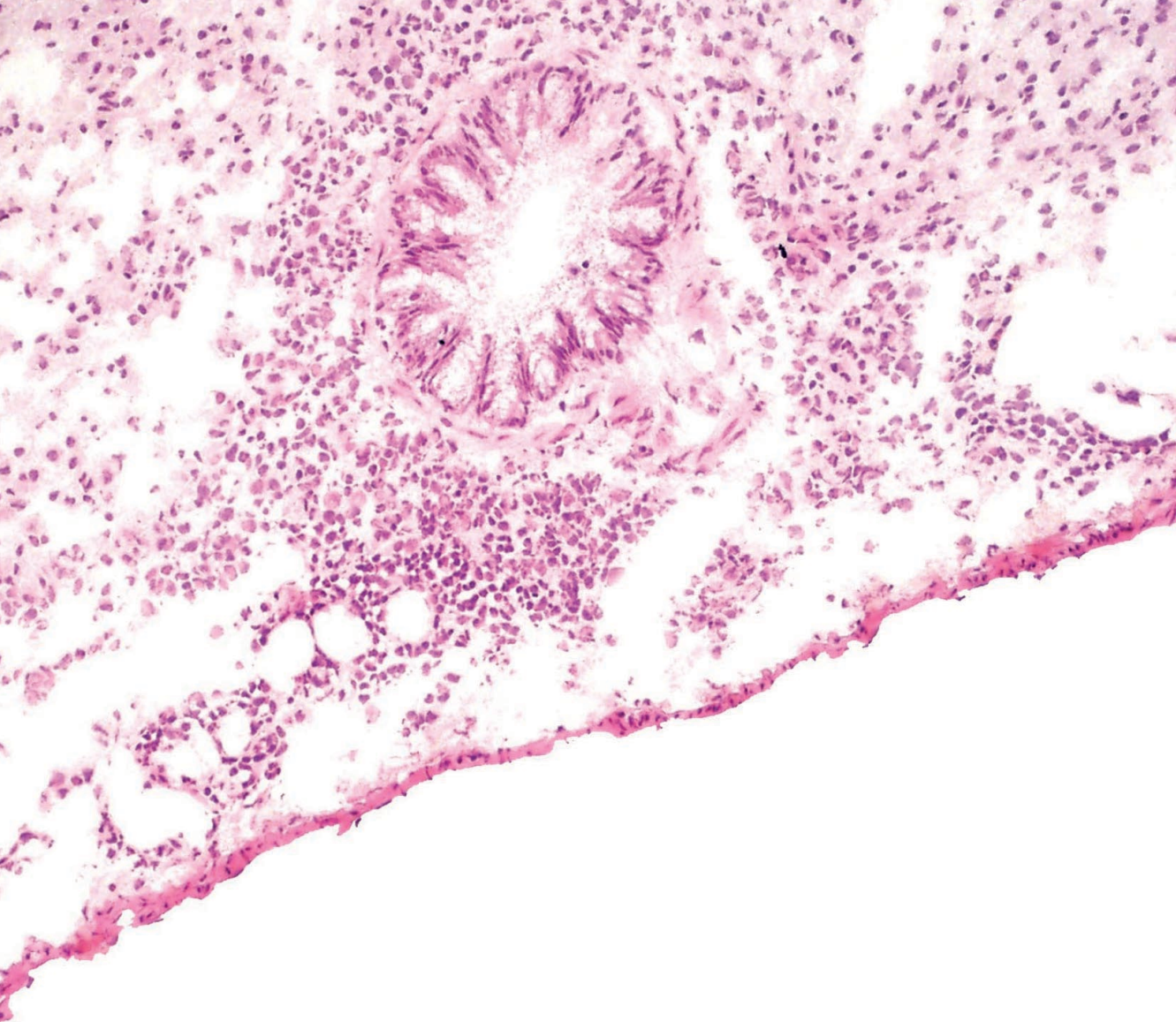
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